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(54) Title: PRODUCTION AND SECRETION OF PROTEINS OF BACTERIAL ORIGIN IN FILAMENTOUS FUNGI

(57) Abstract

The present invention is related to an improved production of bacterial proteins in filamentous fungus, e.g. in *Trichoderma* and *Aspergillus*. The improvement is achieved by constructing expression vectors, which comprise the bacterial protein encoding DNA sequences fused in frame with a DNA sequence encoding a filamentous fungus secretable protein or one or more functional domains of said protein. Filamentous fungus hosts transformed with such expression vectors secrete the desired proteins or enzymes, especially xylanases or cellulases originating from bacteria or more preferably from actinomycetes into the culture medium of the host. The desired proteins or enzymes can be used directly from the culture medium after separation of host cells or recovered and treated using down-stream processes, which are appropriate for the respective application. Xylanases or cellulases from actinomycetes produced using the above expression vectors are most suitable for treating plant derived materials, e.g. in pulp and paper industries.

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Production and Secretion of Proteins of Bacterial Origin in Filamentous Fungi

Field of the Invention

The present invention provides a method for producing proteins or enzymes of bacterial origin in filamentous fungi, such as *Aspergillus* and *Trichoderma*, by transforming said filamentous fungus hosts with an expression vector containing a DNA sequence encoding a secretable fungal protein or one or more functional domains of said protein. Also provided are expression vectors and hosts useful in said method. The transformed hosts produce preparations, especially enzyme preparations useful e.g. for modifying plant biomass properties, especially to reduce the lignin content in enzyme aided bleaching.

Background of the Invention

Thus, the present invention is directed to the production of proteins or enzymes of bacterial origin in filamentous fungi, for example in *Aspergillus* and *Trichoderma*, by using a fusion to a secretable fungal protein or one or more functional domains of said protein to obtain improved secretion of said bacterial protein or enzyme. Preferably, the proteins originate from actinomycetes. The compositions of the invention are useful for e.g. modifying plant biomass properties, especially to reduce the lignin content in enzyme aided bleaching. The invention is also directed to the area of thermostable xylanases that are active at high temperatures and to a method for bleaching with the aid of the enzyme compositions of the invention.

The aim of kraft pulp bleaching is to remove the residual lignin that is left in pulp after kraft cooking. Traditionally, this has been done by using chlorine-containing chemicals. Because of environmental concerns and consumer demands, alternative bleaching technologies have been desired.

The first biotechnical approach to this problem was to attack the lignin directly with lignin degrading enzymes. However, the chemistry of enzymatic lignin degradation seems to be very complicated and difficult to control.

Lignin can be degraded, if the whole microorganism that produces ligninolytic enzymes is

used. However, treatment times are relatively long. For example, treatment times may take days, and the microorganisms need supplemental nutrients to work. It can also be difficult to control the growth of other, undesired, microbes. The use of lignin degradation by isolated ligninolytic enzymes or by microorganisms is the subject of much research. (see, for example, Farrell, R.L. *et al.*, *Lignocellulosics* 305-315 (1992); Jurasek, L., *Lignocellulosics* 317-325 (1992)).

In addition to cellulose and lignin, wood pulp contains hemicellulose. Another approach to reduce the lignin content of pulp is to attack hemicellulose - the third main component of wood. The hemicellulose in native hardwood is mainly xylan, while in softwood the hemicellulose is mainly glucomannans and some xylan. During kraft cooking, part of the xylan is dissolved into the cooking liquor. Towards the end of the cooking period when the alkali concentration decreases, part of the dissolved and modified xylan reprecipitates back onto the cellulose fibre.

In 1986, it was noticed that xylanase pretreatment of unbleached kraft pulp results in a lessened need for chemicals in the bleaching process (Viikari, L. *et al.*. Proceedings of the 3rd Int. Conf. on Biotechnology in the Pulp Paper Ind., Stockholm (1986), pp. 67-69). Xylanase pretreatment of kraft pulp partially hydrolyses the xylan in kraft pulp. The mechanism of how hydrolysis of xylan results in better lignin removal is not fully understood. One frequently suggested possibility is that the pulp structure becomes more porous and this enables more efficient removal of lignin fragments in the subsequent bleaching and extraction stages. Also hydrolysis of the xylan located in the inner parts of the fibre and possibly linked to lignin may have a role. Later, in several laboratories, the xylanase pretreatment was reported to be useful in conjunction with bleaching sequences consisting of Cl_2, ClO_2, H_2O_2, O_2 and O_3. See reviews in Viikari, L. *et al.*, *FEMS Microbiol. Rev. 13*: 335-350 (1994); Viikari, L. *et al.*, *in*: Saddler, J.N., ed., *Bioconversion of Forest and Agricultural Plant Residues*, C-A-B International (1993), pp. 131-182; Grant, R., Pulp and Paper Int. (Sept. 1993), pp. 56-57; Senior & Hamilton, *J. Pulp & Paper* :111-114 (Sept. 1992); Bajpai & Bajpai, *Process Biochem. 27*:319-325 (1992); Orrysko, A., *Biotech. Adv. 11*:179-198 (1993); and Viikari, L. *et al.*, *J. Paper and Timber 73*:384-389 (1991).

As a direct result of the better bleachability of the pulp after such a xylanase treatment, there is a reduction of the subsequent consumption of bleaching chemicals, which when chlorine containing chemicals are used, leads to a reduced formation of environmentally undesired organo-chlorine compounds. Also as a direct result of the better bleachability of pulp after a xylanase treatment, it is possible to produce a product with a higher final

brightness where such brightness would otherwise be hard to achieve (such as totally chlorine free (TCF) bleaching using peroxide). Because of the substrate specificity of the xylanase enzyme, cellulose fibers are not harmed and the strength properties of the product are well within acceptable limits.

A xylanase that is active at an alkaline pH would decrease the need to acidify the pulp prior to xylanase treatment. In addition, the temperatures of many modern kraft cooking and bleaching processes are relatively high, well above the 50 °C that is suitable for many of the commercial bleaching enzymes. Accordingly, a need exists for thermostable xylanase preparations that are stable at alkaline pH values for use in wood pulp bleaching processes.

It is known that actinomycetes, e.g. (*Microtetraspora flexuosa* ATCC35864 and *Thermomonospora fusca* KW3, produce thermostable and alkaline stable xylanases (US 5,437,992 and EP 473 545. The cloning of xylanases has been reported from several bacteria (e.g. Ghangas, G.S. *et al.*, *J. Bacteriol. 171*:2963-2969 (1989); Lin, L.-L., Thomson, J.A., *Mol. Gen. Genet. 228*:55-61 (1991); Shareck, F. *et al.*, *Gene 107*:75-82 (1991); Scheirlinck, T. *et al.*, *Appl Microbiol Biotechnol. 33*:534-541 (1990); Whitehead, T.R., Lee, D.A., *Curr. Microbiol. 23*:15-19 (1991)); and also from *Actinomadura sp.* FC7 (Ethier, J.-F. *et al.*, in: *Industrial Microorganisms: Basic and Applied Molecular Genetics*. R. Baltz *et al.*, Eds, (Proc. 5th ASM Conf. Gen. Mol. Biol. Indust. Microorg., Oct 11-15, 1992, Bloomington, Indiana, poster C25)). It has been proposed by some researchers that the former genus *Actinomadura* should be divided into two genera. *Actinomadura* and *Microtetraspora*, the latter including, e.g. the former *A. flexuosa* (Kroppenstedt *et al.*, *System. Appl. Microbiol. 13*: 148-160 (1990).

The use of hemicellulose hydrolyzing enzymes in different bleaching sequences is discussed in WO 89/08738, EP 383 999, WO 91/02791, EP 395 792, EP 386 888, EP 473 545, EP 489 104 and WO 91/05908, WO 95/34662, WO 95/18219, WO 95/27779, WO 95/34662, WO 95/18219, WO 92/04664 and WO 92/03540. The use of hemicellulolytic enzymes for improved water removal from mechanical pulp is discussed in EP 262 040, EP 334 739 and EP 351 655, DE 4,000,558, WO 92/04664, WO 92/03540. WO 94/21785 and EP 513 140. When the hydrolysis of biomass to liquid fuels or chemicals is considered, the conversion of both cellulose and hemicellulose is essential to obtain a high yield (Viikari *et al.*, "Hemicellulases for Industrial Applications," In: *Bioconversion of Forest and Agricultural Wastes*, Saddler, J., ed., CAB International, USA (1993)). Also, in the feed industry, there is a need to use a suitable combination of enzyme activities to degrade the high ß-glucan and hemicellulose containing substrate.

The efficient and cost-effective production of thermostable xylanases is a problem, because thermostable xylanases originate mainly from relatively unstudied bacteria, which often produce only minimal or very small amounts of xylanase. Further, there is little or no experience of cultivating these microbes in a fermentor or no fermentation processes available. Furthermore, these microbes may be unsuitable for industrial scale production. On the other hand, filamentous fungi like *Aspergillus* and *Trichoderma* are known to produce large quantities of proteins, on an industrial scale. In particular, these fungi have been shown to be suitable for production of homologous or heterologous proteins of fungal origin.

There are very few reports related to the production of proteins or enzymes of bacterial origin in filamentous fungi: the production of endoglucanase from *Cellulomonas fimi* (Gwynne *et al.*, *Bio/Technology 5:* 713-719 (1987); and ß-glucuronidase from *E. coli* (Punt *et al.*, *J. Biotechnol. 17:* 19-34 (1991) have been reported in *A. nidulans*. Of these enzymes, endoglucanase was secreted into the culture medium by *Aspergillus nidulans* in the range of 10-15 mg protein per liter. ß-glucuronidase was only detectable intracellularly.

Many of the studies on heterologous gene expression have concerned mammalian genes (van den Hondel *et al.*, Heterologous gene expression in filamentous fungi, Ed. Bennett and Lasure. *More Gene Manipulations in Fungi* Academic Press, San Diego, U.S.A., pp. 396-428 (1991). So far, the initial yields of eucaryotic enzymes in filamentous fungi have been in a range of tens of mg per liter in shake flask cultivations. In the International patent publication WO 90/15860 secretion of chymosin by *A. niger* var. *awamori* was described using a fusion to the homologous glucoamylase gene. Nyyssönen *et al.*, *Bio/Technology 11*: 591-595 (1993) describes the production of antibody fragments in *Trichoderma reesei*. The best yield of antibody fragments when produced as a fusion to the cellobiohydrolase 1 gene of *T. reesei* was in the range of 40 mg per liter in a shake flask cultivation.

So far the inventors of the present application are not aware of any reports of the production of proteins of bacterial origin in *Trichoderma*.

Summary of the Invention

The invention is directed *inter alia*, to a method of producing, i.e. improved expressing and secreting proteins or enzymes originating from bacteria, especially from actinomycete. The invention, however, is not strictly limited to higher secretion levels. Lesser or greater levels of expression are acceptable. The main purpose of the invention is to provide an alternative

method for producing enzymes originating from actinomycete in filamentous fungi.

The characteristics of the present invention are as set forth in the claims.

The present invention is related to a recombinant expression vector for production of bacterial proteins or enzymes, especially xylanases and cellulases in a filamentous fungal host, especially in *Aspergillus* and *Trichoderma*, most preferably in *T. reesei*. Said vector comprises a promoter operably linked to a DNA sequence of a filamentous fungus secretable protein or one or more functional domains of said protein, which in turn frames the DNA sequence, e.g. SEQ ID NO: 1: or SEQ ID NO: 3:, or equivalents thereof, which encode a bacterial protein, preferably an actinomycetous protein, most preferably *Actinomadura flexuosa* 35 kD (AM35) or 50 kD (AM50) xylanase, i.e. SEQ ID NO: 2: and SEQ ID NO: 3: and equivalents thereof, as well as *Thermomonospora fusca* cellulases, especially the *T. fusca* endocellulase E5.

Also provided are peptide sequences of *A. flexuosa* 35 kDa and 50 kDa protein used for identifying and characterizing the protein sequences. Said peptides are assigned SEQ ID NO: 6:. SEQ ID NO: 7:, SEQ ID NO: 8:, SEQ ID NO: 9: and SEQ ID NO: 10:.

The preferred promoters of the present invention are promoters of a filamentous fungus secretable protein, most preferably a *T. reesei cbh1* promoter or a *A. niger* glucoamylase promoter.

Examples of recombinant expression vector are the plasmids pALK945, pALK948, pALK1021 and pALK1022, which are constructed as shown in figure 17 and contain SEQ ID NO: 11:, SEQ ID NO: 13:, SEQ ID NO: 15: and SEQ ID NO: 17:, respectively and which encode the proteins comprising SEQ ID NO: 12:, SEQ ID NO: 14:, SEQ ID NO: 16:, and SEQ ID NO: 18: or equivalents thereof.

All the proteins comprising SEQ ID NO: 12:, SEQ ID NO: 14:, SEQ ID NO: 16:, and SEQ ID NO: 18: or equivalents thereof have a N-terminal DNA sequence SEQ ID NO: 5:, which is identical with the N-terminal sequence of the wild type *A. flexuosa* xylanase (D-T-T-I-T-Q).

The present invention further provides preparation containing one or more proteins or enzymes, preferably xylanases or cellulases originating from bacteria, especially from actinomycetes in an essentially cell-free culture medium, which can be subjected to suitable down-stream processing methods.

The preparation containing enzymes, especially xylanases or cellulases originating from actinomycetes are obtainable by cultivating hosts transformed with the expression vectors of the present invention. These preparations are useful e.g. for enzyme-aided bleaching, because the enzymes are stable at processing temperatures, when the processing temperatures are in the ranges 50-90 °C, preferably 60-85 °C, most preferably 70-80 °C.

Especially, said preparations are useful for treating paper making pulp and for enzymatical treating of plant biomass.

It is therefore the main object of this invention to produce more efficiently protein or enzymes of bacterial origin in filamentous fungi such as *Aspergillus* or *Trichoderma*. Preferably, the host is *Trichoderma* and the proteins originate from actinomycetes.

Figures

Figure 1 shows the effect of pH on *Actinomadura flexuosa* DSM43186 xylanase activity (culture supernatant) at 50 °C.

Figures 2A, 2B and 2C show the effect of temperature on *A. flexuosa* DSM43186 xylanase activity (culture supernatant) at pH values 6.9, 7.8 and 9.0 and at time points of 0, 30, 60 and 120 minutes. The highest xylanase activity of the whole experiment is described as 100 % and all other activity values are proportional to it.

Figure 2A shows the activities in a temperature of 60 °C.

Figure 2B shows the activities in a temperature of 70 °C.

Figure 2C shows the activities in a temperature of 80 °C.

Figure 3 shows the DEAE Sepharose CL-6B chromatography elution profile of *A. flexuosa* DSM43186 xylanases.

Figure 4A shows the Phenyl Sepharose CL-4B chromatography elution profile of DEAE pool I of Figure 3. The fractions that were combined to provide sample DEPS I/1 are indicated.

Figure 4B shows the Phenyl Sepharose CL-4B chromatography elution profile of DEAE pool II of Figure 3. The fractions that were combined to provide sample DEPS II/1 and

DEPS II/2 are indicated.

Figure 4C shows the Phenyl Sepharose CL-4B chromatography elution profile of DEAE pool III of Figure 3. The fractions that were combined to provide sample DEPS III/1 and DEPS III/2 are indicated.

Figure 5A shows the Coomassie Brilliant Blue protein staining pattern of the various chromatographic pools. Lane 1: molecular weight markers; lane 2: medium; lane 3: DEPS (Pool I/1); lanes 4 and 5: DEPS (Pool II/1 and II/2, respectively); lane 6: empty; lanes 7 and 8: DEPS (Pool III/1 and III/2, respectively). DEPS: Fractions after the DEAE chromatography shown in Figure 3 and the Phenyl Sepharose chromatography shown in Figures 4A-4C.

Figure 5B shows the Western blot analysis of the various chromatographic pools stained in Figure 5A. Polyclonal antiserum raised against the *Thermomonospora fusca* XynA xylanase was used for detection. Lane 1: molecular weight markers; lane 2: medium; lane 3: DEPS (Pool I/1); lanes 4 and 5: DEPS (Pool II/1 and II/2, respectively); lane 6: empty; lanes 7 and 8: DEPS (Pool III/1 and III/2, respectively). DEPS: Fractions after the DEAE chromatography shown in Figure 3 and the Phenyl Sepharose chromatography shown in Figures 4A-4C.

Figure 6A shows the Phenyl Sepharose FF chromatography elution profile of DEAE flow through permeate. The tubes that were combined to provide sample PF1 and PF2 are indicated.

Figure 6B shows the Phenyl Sepharose FF chromatography elution profile of DEAE flow-through concentrate. The tubes that were combined to provide sample KF1, KF2 and KF3 are indicated.

Figure 7A shows the Coomassie Blue protein staining pattern of the various chromatographic pools. Abbreviations are as in Figures 6A and 6B. Lanes 1 and 10: molecular weight markers; lane 2: medium; lane 3: PF1; lane 4: PF2; lane 5: KF1; lane 6: KF2; lane 7: KF3; Lane 8: DEAE flow-through concentrate; Lane 9: Empty.

Figure 7B shows the Western blot analysis of the various chromatographic pools stained for protein in Figure 7A. Polyclonal antiserum raised against the *T. fusca* XynA xylanase was used for detection. Abbreviations are as in Figures 6A and 6B. Lanes 1 and 10: molecular weight markers; lane 2: medium; lane 3: PF1; lane 4: PF2; lane 5: KF1; lane 6: KF2; lane

7: KF3. Lane 8: DEAE flow-through concentrate; Lane 9: empty.

Figure 8 shows the thermostability of purified *A. flexuosa* 35 kDa xylanase (AM35) at 70 °C, pH 6 with and without added bovine serum albumin (BSA). The enzyme sample was incubated in McIlvain's buffer +/-BSA (100 μg/ml). Samples were collected after 0, 2, 6, and 24 hours of incubation and enzyme activity assay was done at pH 6.5, 60 °C.

Figure 9 shows the thermostability of purified *A. flexuosa* 50 kDa xylanase (AM50) at 70 °C, pH 6 with and without added BSA. The enzyme sample was incubated in McIlvain's buffer +/- BSA (100 μg/ml). Samples were collected after 0, 2, 6 and 24 hours of incubation and enzyme activity assay was done at pH 6.5, 60°C.

Figure 10A shows the effect of pH on the activity of the 35 kDa xylanase at 80 °C.

Figure 10B shows the effect of pH on the activity of the 50 kDa xylanase at 60 °C, (closed squares), 70 °C (open squares) and 80 °C (closed circles).

Figure 10C shows the effect of pH on the activity of the 35 kDa (closed squares) and the 50 kDa (open squares) xylanases at 60 °C with 60 minutes incubations.

Figure 11 shows the effect of temperature on the activity of the 35 kDa (closed squares) and the 50 kDa (open squares) at pH 7 with 60 minutes incubations.

Figure 12 is a map of plasmid pALK185 (4.5 kb), containing *T. fusca* xylanase gene (*xyn*A).

Figure 13 shows the DNA sequence and the amino acid sequence of 1375 bps of *A. flexuosa* DSM43186 35 kDa xylanase.

Figure 14 shows the DNA sequence and amino acid sequence of 1864 bps of *A. flexuosa* DSM43186 50 kDa xylanase.

Figure 15A shows a homology comparison at the amino acid level between the AM50-peptide derived from the 1864 bps insert and the *Actinomadura* sp. FC7 xylanase II (accession no. U08894) gene. The figure shows that there was 70.7% identity in a 434 amino acid overlap.

Figure 15B shows a homology comparison at the amino acid level between the

AM50-peptide derived from the 1864 bps insert and the *Streptomyces lividans* xylanase A (*xlnA*) gene (accession no. M64551). The figure shows that there was 70.3% identity in a 489 amino acid overlap.

Figure 16 shows the expression cassette pALK193 (12 kb) containing the expression cassette for production of *T. fusca* xylanase XynA in *T. reesei*.

Figure 17 is a map of plasmid pALK1022 (13 kb) containing the expression cassette for production of *A. flexuosa* 35 kDa xylanase in *T. reesei*.

Figure 18 is a map of plasmid pALK1055 (4.5 kb) containing the gene for *A. flexuosa* 35 kDa xylanase.

Figure 19 shows the DNA sequence SEQ ID NO: 11:, SEQ ID NO: 13:, SEQ ID NO: 15: and SEQ ID NO: 17 as well as the corresponding amino acid sequences SEQ ID NO: 12:, SEQ ID NO: 14:, SEQ ID NO: 16: and SEQ ID NO: 18:, which comprise the fusions between the *man1* core/hinge and the *am35* gene for pALK945, pALK948, pALK1021 and pALK1022.

Figure 20 shows the thermal stability of xylanase activity from culture supernatants of a *A. flexuosa* DSM43186 and chosen *T. reesei* transformants producing *A. flexuosa* 35 kDa xylanase. Samples from the culture supernatants were incubated at 70 °C, pH 7 in McIlvain's buffer. (BSA was added to 100 μg/ml) for 0, 15, 30, 60 and 120 minutes after which xylanase activities from the sample were determined at 70 °C, pH 7 (5 minutes incubation).

Figure 21 shows a Western blot analysis of culture supernatants from transformants producing *A. flexuosa* AM35 xylanase. Polyclonal antiserum raised against the purified ß-mannanase (pI 5.4) of *T. reesei* RutC30 was used for detection. Lane 1: prestained low molecular weight marker (Bio-Rad, U.S.A.); Lanes 2-3: purified 53 kDa ß-mannanase protein sample; Lane 4: culture medium of MANI core producing strain, ALKO 3620/pALK1010/24. Lane 5: culture medium of the transformation host strain ALKO3620; Lanes 6-10: Culture media of the transformants ALKO3620/pALK945/8, ALKO3620/pALK945/6, ALKO3620/pALK948/7, ALKO3620/pALK1022/29 and ALKO3620/pALK1021/4, respectively.

Figure 22 shows a Western blot analysis of culture supernatants from transformants producing *A. flexuosa* xylanase. Polyclonal antiserum raised against the purified 35 kDa

xylanase of *A. flexuosa* was used for detection. Lane 1: prestained low molecular weight marker (Bio-Rad, U.S.A.); Lane 2: purified 35 kDa xylanase of *A. flexuosa* DSM43186; Lane 3: 53 kDa ß-mannanase protein sample; Lane 4: culture medium of the MANI core producing strain, ALKO3620/pALK1010/24; Lane 5: culture medium of the transformation host ALKO3620; Lanes 6-10: culture media of the transformants ALKO3620/pALK945/8, ALKO3620/pALK945/6, ALKO3620/pALK948/27, ALKO3620/pALK1022/29 and ALKO3620/pALK1021/4, respectively.

Figure 23A: Western-blot of growth medium samples from a Fed-batch fermentation of ALKO3620/pALK945/8. Polyclonal antibody raised against the purified *A. flexuosa* 35 kDa xylanase. Lane 1 and 9: prestained molecular mass standards (LMW, Bio-Rad); Lane 3 to 8: growth medium samples after 7, 6, 5, 4, 3 and 2 days fermentation times, respectively; Lane 2: final sample after 7.3 days fermentation.

Figure 23B: Western-blot of growth medium samples from a laboratory fermentation of ALKO3620/pALK945/6. Polyclonal antibody raised against the purified *A. flexuosa* 35 kDa xylanase. Lane 1: prestained molecular mass standards (LMW, Bio-Rad); Lane 2 to 6: growth medium samples after 1, 2, 3, 4 and 5 days fermentation times, respectively.

Figure 24: SDS-PAGE of purified recombinant *A. flexuosa* 35 kDa xylanases and wild-type *A. flexuosa* 35 kDa xylanase. Lanes 1 and 8: Prestained molecular mass standards (Bio-Rad); Lane 3: purified wild-type 35 kDa xylanase; Lane 4: purified 33.4 kDa xylanase; Lane 5: purified 23.8 kDa xylanase; Lane 6: purified 22 kDa xylanase; Lane 2 and 7: empty.

Figure 25: Temperature and pH dependence of purified recombinant and wild-type *A. flexuosa* 35 kDa xylanases. Incubations were performed at temperatures and pH values indicated for 60 min with 1 % (w/v) birch xylan (Roth 7500) as substrate.

Deposits

Plasmid pALK923, pALK938, pALK939, pALK940, pALK941 and pALK1056 were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1 b, D-38124 Braunschweig, Germany assigned accession numbers DSM9322, DSM9899, DSM9900, DSM9901, DSM9902 and DSM9903, respectively. pALK923 was deposited on July 27, 1994, and pALK938-941 and pALK1056 were deposited on April 3, 1995.

Plasmids pALK927 and pALK928 were deposited at the DSM on September 27, 1994, and assigned accession numbers DSM9447 and DSM9448, respectively.

Detailed Description of the Preferred Embodiments

I. Definitions

In the description that follows, a number of terms used in recombinant DNA technology are extensively utilized. In order to provide a clearer and consistent understanding of the specification and claims, including the scope to be given such terms, the following definitions are provided.

Cloning vehicle. A plasmid or phage DNA or other DNA sequence (such as a linear DNA) which provides an appropriate nucleic acid environment for the transfer of a gene of interest into a host cell. The cloning vehicles of the invention may be designed to replicate autonomously in prokaryotic and eukaryotic hosts. In fungal hosts such as *Trichoderma*, the cloning vehicles generally do not autonomously replicate and instead, merely provide a vehicle for the transport of the gene of interest into the *Trichoderma* host for subsequent insertion into the *Trichoderma* genome. The cloning vehicle may be further characterized by one or a small number of endonuclease recognition sites at which such DNA sequences may be cut in a determinable fashion without loss of an essential biological function of the vehicle, and into which DNA may be spliced in order to bring about replication and cloning of such DNA. The cloning vehicle may further contain a marker suitable for use in the identification of cells transformed with the cloning vehicle. Suitable marker genes are for example antibiotic resistance providing marker genes, but other useful markers exist as well, e.g. biocide resistance providing genes as well as heavy metals, such as copper resistance providing genes. Alternatively, such markers may be provided on a cloning vehicle which is separate from that supplying the gene of interest using the so called cotransformation system. The word "vector" is sometimes used for "cloning vehicle."

Expression vehicle. An expression vehicle or vector is similar to a cloning vehicle but it is capable of expressing a gene of interest, after transformation into a desired host. When a fungal host is used, the gene of interest is preferably provided to a fungal host as part of a cloning or expression vehicle that integrates into the fungal chromosome. Sequences which derive from the cloning vehicle or expression vehicle may also be integrated with the gene of interest during the integration process. For example, in *T. reesei*, the gene of interest can be directed to the *cbh1* locus.

The gene of interest may preferably be placed under the control of (i.e., operably linked to) certain control sequences such as promoter sequences provided by the vector (which integrate with the gene of interest). If desired, such control sequences may be provided by the host's chromosome as a result of the locus of insertion.

Expression control sequences on an expression vector will vary depending on whether the vector is designed to express a certain gene in a prokaryotic or eukaryotic host (for example, a shuttle vector, may provide a gene for selection in different microbial hosts) and may additionally contain transcriptional elements such as, enhancer elements, termination sequences, and/or translational initiation and termination sites.

Homologous. By an enzyme "***homologous***" to a host of the invention is meant that an untransformed strain of the same species as the host species naturally produces some amount of the native protein; by a gene "homologous" to a host of the invention is meant a gene found in the genome of an untransformed strain of the same species as the host species. By an enzyme "***heterologous***" to a host of the invention is meant that an untransformed strain of the same species as the host species does not naturally produce some amount of the native protein; by a gene "heterologous" to a host of the invention is meant a gene not found in the genome of an untransformed strain of the same species as the host species.

Xylanase. As used herein, a xylanase is a hemicellulase that cuts the ß-1,4 bonds within the xylosic chain of xylan, (xylan is a polymer of D-xylose residues that are joined through ß-1,4 linkages). Xylanase activity is synonymous with xylanolytic activity. More specifically xylanolytic activity means the an activity similar with or identical to the xylanolytic activity of *A. flexuosa* 35 kDa (AM35) and *A.flexuosa* 50 kDa (AM50), the characteristics, e.g. the thermostability are more specifically described and defined in the detailed description and examples.

By an amino acid sequence that is an "***equivalent***" of a specific amino acid sequence is meant an amino acid sequence that is not identical to the specific amino acid sequence, but rather contains at least some amino acid changes (deletion, substitutions, inversions, insertions, etc.) that do not essentially affect the biological activity of the protein as compared to a similar activity of the specific amino acid sequence, when used for a desired purpose. Preferably, an "equivalent" amino acid sequence contains at least 85%-99% identity at the amino acid level to the specific amino acid sequence, most preferably at least 90% and in an especially highly preferable embodiment, at least 95% identity, at the amino

acid level. In the case of larger deletions, e.g. the removal of the tail region, the comparison is performed to the amino acid sequence of the corresponding area in the original sequence.

Functional Domains. The term in relation to a secretable fungal protein includes e.g. the secretion signal (signal sequence or signal sequence and prosequence of the secretable protein or part of the protein, which contains sequences that permit the proteins or fusion constructions to be secreted. In other words, the term "functional domain" means a region of a DNA sequences which encodes a specific region of a protein. In this invention the term "functional domain" includes, in addition, to the regions of the DNA sequence encoding the amino acid sequence responsible for the catalytic or enzymatic function, other specific domains with other functions, e.g. a binding function. The binding function is responsible for the binding to respective protein, e.g. to xylan or cellulose. The specific domain can also be a folding domain, which is responsible for the tertiary structure of the protein, e.g. it encodes an α-helical or ß-sheet structure of a protein or a combination thereof. The functional domain can also be responsible for the immunological activity of the protein. Thus, the "functional domain" might comprise a secretion signal or the core sequence or a sequence responsible for the folding of the protein as set forth above. Said functional domains can be totally separate from each other and are responsible for the "**biological**" activity of the protein. By the "biological" activity of a xylanase amino acid sequence of the invention is meant the enzymatic, functional, folding or binding activity or a combination of said activities.

Preparation or enzyme preparation. By "preparation or enzyme preparation" is meant a composition containing proteins or enzymes which are present in the culture medium and from which the host cells have been removed after the cultivation or fermentation has been completed. The preparation or enzyme preparation can be further processed by down-stream-processing methods, which are appropriate for the application of the protein or enzyme. The proteins or enzymes can be either partially or completely isolated and purified. For bulk use the preparation or enzyme preparation is usually subjected to filtration and/or centrifugation to remove the host cells from the spent culture medium. Thus the term "**preparation or enzyme preparation**" in the present invention means crude enzyme preparations for bulk use, but also proteins or enzymes, which can be highly purified for special reasons.

By a host that is "***substantially incapable***" of synthesizing one or more enzymes is meant a host in which the activity of one or more of the listed enzymes is depressed, repressed, deficient, or absent when compared to the wild-type.

Enzyme-aided bleaching. By "enzyme-aided bleaching" is meant the extraction of residual lignin from paper making pulp after the action of hemicellulose degrading enzymes with or without lignin degrading enzymes. The removal or extraction of lignin may be restricted by hemicelluloses either physically (through reprecipitation onto the fibre surface during cooking) or chemically (through lignin-carbohydrate complexes). The hemicellulase activity partially degrades the hemicellulose, which enhances the extractability of lignins by conventional bleaching chemicals (like chlorine, chlorine dioxide, peroxide, etc.) (Viikari *et al.*, "Bleaching with Enzymes" in *Biotechnology in the Pulp and Paper Industry*, Proc. 3rd Int. Conf., Stockholm, pp. 67-69 (1986); Viikari *et al.*, "Applications of Enzymes in Bleaching" in *Proc. 4th Int. Symp. Wood and Pulping Chemistry*, Paris, Vol. 1, pp. 151-154 (1987); Kantelinen *et al.*, "Hemicellulases and their Potential Role in Bleaching" in *International Pulp Bleaching Conference, Tappi Proceedings*, pp. 1-9 (1988)). The advantage of this improved bleachability is a lower consumption of bleaching chemicals and lower environmental loads or higher final brightness values. In the past, this has often been referred to as biobleaching.

II. Genetic Engineering of the Hosts of the Invention

The problem of producing bacterial proteins, preferably xylanases in a cost-effective manner in a large scale is solved by producing the proteins in filamentous fungi, e.g. *Aspergillus* or *Trichoderma*. The process for efficiently producing bacterial proteins in filamentous fungi is facilitated through the cloning of genetic sequences that encode the desired bacterial protein activity and through the expression of such genetic sequences in filamentous fungi. As used herein, the term "genetic sequences" is intended to refer to a nucleic acid molecule (preferably DNA). Genetic sequences that encode the desired proteins are derived from a variety of bacterial sources. These sources include actinomycetous DNA, cDNA, synthetic DNA and combinations thereof, preferably actinomycetous DNA, cDNA, synthetic DNA and combinations thereof encoding xylanase, most preferably *Actinomadura* genomic DNA, cDNA, synthetic DNA and combinations thereof. Vector systems may be used to produce hosts for the production of the enzyme preparations of the invention. Such vector construction (a) may further provide a separate vector construction (b) which encodes at least one desired gene to be integrated to the genome of the host and (c) a selectable marker coupled to (a) or (b). Alternatively, a separate vector may be used for the marker.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains expression control sequences which contain transcriptional regulatory

information and such sequences are "operably linked" to the nucleotide sequence which encodes the polypeptide.

An operable linkage is a linkage in which a sequence is connected to a regulatory sequence (or sequences) in such a way as to place expression of the sequence under the influence or control of the regulatory sequence. Two DNA sequences (such as a protein encoding sequence and a promoter region sequence linked to the 5' end of the encoding sequence) are said to be operably linked if induction of promoter function results in the transcription of the protein encoding sequence mRNA and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the expression regulatory sequences to direct the expression of the mRNA, antisense RNA, or protein, or (3) interfere with the ability of the template to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence.

The precise nature of the regulatory regions needed for gene expression may vary between species or cell types, but shall in general include, as necessary, 5' non-transcribing and 5' non-translating (non-coding) sequences involved with initiation of transcription and translation respectively.

Expression of the protein in the transformed hosts requires the use of regulatory regions functional in such hosts. A wide variety of transcriptional and translational regulatory sequences can be employed. In eukaryotes, where transcription is not linked to translation, such control regions may or may not provide an initiator methionine (AUG) codon, depending on whether the cloned sequence contains such a methionine. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis in the host cell.

As is widely known, translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes the protein, or a functional derivative thereof, does not contain any intervening codons which are capable of encoding a methionine. The presence of such codons results either in a formation of a fusion protein (if the AUG codon is in the same reading frame as the protein encoding DNA sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the protein encoding sequence).

In a preferred embodiment, a desired protein is secreted into the surrounding medium due to the presence of a secretion signal sequence. If a desired protein does not possess its own signal sequence, or if such signal sequence does not function well in the host, then the protein's coding sequence may be operably linked to a signal sequence homologous or heterologous to the host. The desired coding sequence may be linked to any signal sequence which will allow secretion of the protein from the host. Such signal sequences may be designed with or without specific protease sites such that the signal peptide sequence is amenable to subsequent removal. Alternatively, a host that leaks the protein into the medium may be used, for example a host with a mutation in its membrane.

If desired, the non-transcribed and/or non-translated regions 3' to the sequence coding for a protein can be obtained by the above-described cloning methods. The 3'-non-transcribed region may be retained for its transcriptional termination regulatory sequence elements: the 3'-non-translated region may be retained for its translational termination regulatory sequence elements, or for those elements which direct polyadenylation in eukaryotic cells.

The vectors of the invention may further comprise other operably linked regulatory elements such as enhancer sequences.

In a preferred embodiment, genetically stable transformants are constructed whereby a desired protein's DNA is integrated into the host chromosome. The coding sequence for the desired protein may be from any source. Such integration may occur *de novo* within the cell or, in a most preferred embodiment, be assisted by transformation with a vector which functionally inserts itself into the host chromosome, for example, DNA elements which promote integration of DNA sequences in chromosomes.

Cells that have stably integrated the introduced DNA into their chromosomes are selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector in the chromosome, for example the marker may provide biocide resistance, e.g., resistance to antibiotics, or heavy metals, such as copper, or the like. The selectable marker gene can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection.

Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Once the vector or DNA sequence containing the construct(s) is prepared for expression, the DNA construct(s) is introduced into an appropriate host cell by any of a variety of suitable means, including transformation as described above. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of transformed cells. Expression of the cloned gene sequence(s) results in the production of the desired protein, or in the production of a fragment of this protein. This expression can take place in a continuous manner in the transformed cells, or in a controlled manner.

Accordingly, the bacterial protein encoding sequences may be operably linked to any desired vector and transformed into a selected filamentous fungi host, preferably *Trichoderma* host, so as to provide for expression of such proteins in that host.

To facilitate secretion of the bacterial protein, the bacterial protein encoding sequences may be fused in frame to other sequences so as to construct DNA encoding a fusion protein. For example, a recombinant vector encoding a xylanase gene from bacterial origin is fused with the sequence of a *Trichoderma* cellulase or hemicellulase, or one or more functional domains of said cellulase or hemicellulase as described in US 5,298,405, WO 93/24622 and in Stålbrand et al., *Appl. Environ. Microbiol. 61*:1090-1097 (1995), each incorporated herein by reference. Especially, the enzyme is selected from the group consisting of cellobiohydrolases (CBHI and CBHII), endoglucanases (EGI and EGII), xylanases (XYLI and XYLII) and mannanase (MANI), or a domain thereof, such as the functional domain signal, preprosequence or the core sequence. MANI has the same domain structure as that of the cellulases: a core domain, containing the active site, a hinge domain containing a serine-threonine rich region, and a tail, containing the binding domain.

If a xylanase gene of bacterial origin is fused in frame to an *Aspergillus* sequence, the sequence is selected from the group consisting of secretable proteins like *A. niger* or *A. niger* var. *awamori* glucoamylase or one or more functional domains of said secretable proteins.

Fusion peptides can be constructed that contain an N-terminal mannanase or cellobiohydrolase or endoglucanase core domain or the core and the hinge domains from the same, fused to the *Actinomadura* xylanase sequence. The result is a protein that contains N-terminal mannanase or cellobiohydrolase or endoglucanase core or core and hinge regions, and a C-terminal *Actinomadura* xylanase. The fusion protein contains both the mannanase or cellobiohydrolase or endoglucanase and xylanase activities of the various domains as provided in the fusion construct. A further alternative is to use a gene coding

for a modified or inactive mannanase or cellobiohydrolase or endoglucanase core domain or the core and hinge domains from the same, fused to *Actinomadura* xylanase sequences. The resulting fusion protein then contains the modified or inactive enzyme domain fused to a desired bacterial sequence.

It should be noted, however, that the whole core region may not be necessary to obtain secretion of the desired fusion protein. A shorter fragment of this domain may also be used, particularly a fragment of the domain containing secretory signals for the protein of interest or a sequence of a specific domain.

Fusion proteins can also be constructed such that the mannanase or cellobiohydrolase or endoglucanase tail or a desired fragment thereof, is included, placed before the *Actinomadura* xylanase sequence, especially so as to allow use of a nonspecific protease site in the tail as a protease site for the recovery of the xylanase sequence from the expressed fusion protein. Alternatively, fusion proteins can be constructed that provide for a protease site in a linker that is placed before the *Actinomadura* xylanase, with or without tail sequences.

Accordingly, this invention results in the production and secretion of bacterial enzymes in filamentous fungi. The bacterial protein or enzyme is encoded as a fusion to a gene of a secretable fungal protein, thereby resulting in a high level of expression and secretion. The improvement in secretion of bacterial enzymes is more than ten-fold compared to the production and secretion of bacterial proteins without a fusion of the bacterial protein encoding gene to a fungal gene encoding a secretable protein. When a mammalian protein like chymosin is produced as a fusion protein in *Aspergillus*, the level of production has been only 10-20% of the production levels of this invention (WO 90/15860). Similarly, the production level of immunoglobulins in *Trichoderma* has been only 10-20% of the production levels of this invention (WO 92/01797).

There are very few reports related to the production of proteins or enzymes of bacterial origin in filamentous fungi: the production of endoglucanase from *Cellulomonas fimi* (Gwynne *et al.*, *Bio/Technology 5:* 713-719 (1987); and ß-glucuronidase from *E. coli* (Punt *et al.*, *J. Biotechnol. 17:* 19-34(1991) have been reported in *A. nidulans*. Of these enzymes, endoglucanase was secreted into the culture medium in the range of 10-15 mg protein per liter. ß-glucuronidase was only detectable intracellularly.

Many of the studies on heterologous gene expression have concerned mammalian genes (van den Hondel *et al.*, Heterologous gene expression in filamentous fungi, Ed. Bennett

and Lasure. *More Gene Manipulations in Fungi* Academic Press, San Diego, U.S.A., pp. 396-428 (1991). So far, the initial yields of eucaryotic enzymes in filamentous fungi have been in a range of tens of mg per liter in shake flask cultivations. In the International patent publication WO 90/15860 secretion of chymosin by *A. niger* var. *awamori* was described using a fusion to the homologous glucoamylase gene. Nyyssönen *et al.*, *Bio/Technology 11*: 591-595 (1993) describes the production of antibody fragments in *Trichoderma reesei*. The best yield of antibody fragments when produced as a fusion to the cellobiohydrolase 1 gene of *T. reesei* in the range of 40 mg per liter in a shake flask cultivation.

III. The Enzyme Preparations of the Invention

According to the invention, there is provided a method for producing enzymes of bacterial origin. These enzymes are synthesized as fusion proteins. The carrier protein in the fusion is a fungal protein or one or more functional domains of said protein that is readily secreted from the host.

The enzyme compositions of the invention satisfy, e.g. requirements of specific needs in various applications in the pulp and paper industry. These applications include, e.g. enzyme-enhanced bleaching of paper making pulp, enzymatic fiberization during beating, enzymatic increase of drainage rates and ink removal of secondary fibre as well as enzymatic pitch removal.

The invention is applied to produce enzyme preparation partially or completely deficient in cellulolytic activity (that is, in the ability to degrade cellulose) and enriched in xylanases desirable for enzyme-enhanced bleaching of chemical pulp. By deficient in cellulolytic activity is meant a reduced, lowered, depressed, or repressed capacity to degrade cellulose. Such cellulolytic deficient preparations, and the making of same by recombinant DNA methods, are described in US 5,298,405, incorporated herein by reference. As described herein, xylanases may be provided directly by the hosts of the invention (the hosts themselves are placed in the wood processing medium). Alternatively, used medium from the growth of the hosts, or purified enzymes therefrom, can be used. Further, if desired activities are present in more than one recombinant host, such preparations may be isolated from the appropriate hosts and combined prior to use in the method of the invention.

To obtain the enzyme preparations of the invention, the recombinant hosts described above having the desired properties (that is, for example, hosts capable of expressing large quantities of the desired xylanase enzymes and optionally, those which are substantially

incapable of secreting one or more cellulase enzymes) are cultivated under suitable conditions, the desired protein or enzymes are secreted from the hosts into the culture medium, and the enzyme preparation is recovered from said culture medium by methods known in the art.

The enzyme preparation is the culture medium with or without the native or transformed host cells, or is recovered from the same by the application of methods well known in the art. However, because the xylanase enzymes are secreted into the culture media and display activity in the ambient conditions of the hemicellulolytic liquor, it is an advantage of the invention that the enzyme preparations of the invention may be utilized directly from the culture medium with no further purification. If desired, such preparations may be lyophilized or the enzymatic activity otherwise concentrated and/or stabilized for storage. The enzyme preparations of the invention are very economical to provide and use because (1) the enzymes may be used in a crude form; isolation of a specific enzyme from the culture fluid is unnecessary and (2) because the enzymes are secreted into the culture medium, only the culture medium need be recovered to obtain the desired enzyme preparation; there is no need to extract an enzyme from the hosts.

If desired, an expressed protein may be further purified in accordance with conventional conditions, such as extraction, precipitation, chromatography, affinity chromatography, electrophoresis, or the like.

IV. Identification and Isolation of **Actinomadura flexuosa** *Xylanases*

Two xylanases have been identified, purified and cloned from *Actinomadura flexuosa*. Both of these xylanases have a pH optimum and thermostability that are desirable for the enzyme-aided bleaching of wood pulp. One of these xylanases has a molecular weight of about 35 kDa (AM35) and the other has a molecular weight of about 50 kDa (AM50).

The optimal temperature range for *A. flexuosa* xylanases in crude preparations is 70-80 °C at pH 6-7. At pH 8, the optimum temperature range of this xylanase preparation is 60-70 °C. This is useful in kraft pulp bleaching because after kraft cooking, the pH of the pulp is alkaline.

In purified preparations, AM35 retains 80% of its activity, and AM50 retains 90% of its activity after 24 hours when incubated in the presence of BSA at pH 6.0 and 70 °C. At 80 °C, both AM35 and AM50 are most active at pH 6 but both exhibit a broad activity plateau between pH 5 - pH 7, wherein about 80% of the activity is retained.

For the isolation of AM35 and AM50, the host *A. flexuosa* is available as depository accession number DSM43186 from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany. Both forms can be purified by passage through a series of chromatographic columns. A first purification step by DEAE Sepharose CL-4B retains about half of the xylanase activity when the sample is applied at pH 8.6-9 in 12.5 mM Na_2HPO_4; the other half is found in the flow through.

Elution of the bound xylanase activity with a salt gradient results in an elution of a sharp, earlier eluting peak of activity and a broad, later eluting peak of activity. The sharp, earlier eluting peak retains its homogeneity when subjected to phenyl Sepharose CL-4B chromatography. Samples taken from the later, broad peak of activity separate into at least two peaks when subjected to Phenyl Sepharose CL-4B chromatography. There is only weak cross-reactivity of these later eluting xylanases with a polyclonal antibody directed against *Thermomonospora fusca* xylanase.

By SDS-PAGE, the molecular weight of the xylanase in these pools from the DEAE retentate was about 50 kDa, while the molecular weights of the xylanases in the DEAE flow through was 30, 35, 40 and 50 kDa. Thus, *A. flexuosa* contains four to five xylanase protein bands.

V. Enzyme-aided Bleaching using the Actinomadura flexuosa *Xylanases*

The present invention comprehends a method for enzymatically treating plant biomass under conditions of high temperature of 50-90 °C and pH 5-8, and especially 60-85 °C, pH 6-7 and most preferably 70-80 °C and pH 7.0 for one to two hours. In a preferred embodiment, plant biomass is treated with xylanases that are able to hydrolyze xylan chains in wood pulp at neutral or moderately alkaline pH and high temperature. Alternatively, enzyme treatment can be combined to chelating stages (metal removal stages) in which high temperatures but low pH values (4-6) are typically used.

Wood pulp is a composite material consisting primarily of a matrix of cellulose, hemicellulose, and lignin. A common procedure for wood pulp production is chemical pulping. One typical mode of chemical pulping is alkaline sulphate cooking, so called kraft cooking. Under the process conditions (high temperatures and high alkalinity), the cooking chemicals extract lignin out of the pulp. However, not all of the lignin is removed during cooking, but part of it, (about 5%), remains in the pulp. This residual lignin has to be removed in order to get pulp suitable for paper production.

Many processes have been developed for the removal of residual lignin. Typically, the wood pulp is treated with chlorine or other toxic chemicals in order to remove the lignin component and provide a bleached pulp. However, the toxic by-products of this chemical treatment have a negative impact upon the health and stability of the environment into which they are released. Consequently, there is a great need for developing alternative, more environmentally protective techniques for pulp bleaching. Treatment of the cooked pulp with enzymes that partially degrade hemicellulose, e.g., xylan, in the pulp, modifies the pulp so that the lignin becomes easier to extract or remove. This leads to improved bleachability which in turn gives the advantages of lower bleaching chemical consumption and lower environmental loads and/or higher final brightness.

Under the method of the present invention, an enzyme-aided bleaching technique is developed whereby thermostable and neutral xylanases can be used in such conditions that the need to adjust the pH and temperature after the cooking step is decreased or eliminated.

In a preferred embodiment, the process of the invention is carried out *in vitro* in wood pulp. The process involves placing the enzyme preparation, culture medium, or concentrated mixture containing xylanase into contact with the wood pulp. Routine calculations enable those in the art to determine the dosage of the xylanase enzyme used, treatment pH and temperature and other parameter variables.

The method of the present invention may be applied alone or as a supplement to other treatments that improve the removal of lignin from wood pulp. In a preferred embodiment, the present invention is used to enhance the bleachability of wood pulps, especially chemical pulps.

In a preferred embodiment, the xylanases used in the methods of the invention are preferably those of *A. flexuosa*, and especially the 35 kDa and/or 50 kDa xylanases of *A. flexuosa*. Especially, culture medium that contains the enzymes secreted as a result of the growth of the cells are useful in the methods of the invention, as are the culture medium that can be provided by a recombinant host that has been transformed with the xylanase encoding genes of the invention.

VI. Preferred and Further Embodiments and Applicability of the Invention

The invention describes an improved method of expressing and secreting proteins or enzymes originating from bacteria, especially from actinomycetes in filamentous fungi by

using fusion protein techniques. More than 50-fold greater production and secretion levels than those observed in the original actinomycete strains have been obtained. The production and secretion levels were more than 10-fold higher than those previously observed when producing heterologous (mammalian or bacterial) enzymes in filamentous fungi. The invention, however, is not strictly limited to higher secretion levels. Lesser or greater levels of expression are acceptable. The main purpose of the invention is to provide an alternative method for producing enzymes originating from bacteria, especially actinomycete in filamentous fungi.

Thus, the invention is related to a recombinant expression vector for production of bacterial proteins in a filamentous fungal host. Said vector comprises a promoter operably linked to a DNA sequence of a filamentous fungus secretable protein or one or more functional domains of said protein, which is fused in frame with a DNA sequence encoding a bacterial protein.

Generally, the filamentous fungus secretable protein encoding DNA sequence encodes an enzyme, such as cellulases or hemicellulases. The enzymes can be homologous or heterologous to the secreting filamentous fungus, e.g. *Aspergillus* or *Trichoderma*. In the most preferred embodiments of the invention the DNA sequence encoding the filamentous fungus secretable protein is an enzyme, which is known to be secreted in significant amounts in *T. reesei*.

Useful proteins or enzymes to provide the objectives of the invention are for example *Aspergillus* glucoamylase, *Aspergillus* α-amylase, *Trichoderma* cellulase, *Trichoderma* hemicellulase, *Trichoderma* glucoamylase, *Hormoconis* glucoamylase, *Chaetomium* xylanase, and *Melanocarpus* cellulase, but the most preferred filamentous fungus secretable proteins are the following enzymes, which are homologous to *Trichoderma*, i.e. cellobiohydrolases (CBHI and CBHII), endoglucanases (EGI and EGII), xylanases (XYLI and XYLII) and mannanase (MANI).

The amino acid and DNA sequences of the enzymes mentioned above are well known from literature and some are described in the examples of the present invention below.

The DNA and amino acid sequences for *Hormoconis resinae* glucoamylase are described in Joutsjoki *et al., Curr. Genet. 24*:223-228 (1993); *Chaetomium* xylanase is described in International Patent Application PCT/FI96/00671; and *Melanocarpus* cellulase in International Patent Application PCT/FI96/00550, which citations hereby are incorporated by reference into the description of the invention. The skilled person can by using said

sequences construct a multitude of alternative useful expression vectors according to the principals set forth in this application.

Because the main objective of the invention is the production of enzymes originating from bacteria or actinomycete, the recombinant expression vector of the present invention comprises a DNA sequence encoding an enzyme originating from a bacterium, preferably a xylanase or cellulase originating from an actinomycete.

Examples of such useful DNA sequences are those obtainable from *A. flexuosa* the DNA sequence SEQ ID NO: 1: encoding the amino acid sequence of SEQ ID NO: 2: or the DNA sequence SEQ ID NO: 3: encoding the amino acid sequence of SEQ ID NO: 4: or any equivalents of said amino acid sequences, wherein said equivalents have an xylanolytic activity similar to that of SEQ ID NO: 2: and SEQ ID No: 4:. Also some peptides of the *A. flexuosa* xylanases assigned SEQ ID NO: 12:, SEQ ID NO: 14:, SEQ ID NO: 16:, and SEQ ID NO: 18: are described below in the examples.

Useful DNA sequences can be found e.g. in the plasmids pALK923 (DSM9322), pALK938 (DSM9899), pALK939 (DSM9900, pALK940 (DSM9901), pALK941 (DSM9902) and pALK1056 (DSM9903) that encode the *Actinomadura flexuosa* AM35 xylanase and plasmids pALK927 (DSM9447) and plasmid pALK928 (DSM9448) that encode the *Actinomadura flexuosa* AM50 xylanase.

The recombinant expression vector of the present invention can alternatively contain a DNA sequence, which encodes *Thermomonospora fusca* cellulases, especially the *T. fusca* endocellulase EV (Lao *et al., J. Bacteriol. 173:* 3397-3407 (1991)).

The preferred promoters in the recombinant DNA expression vectors of the present invention are those of a filamentous fungus secretable protein, most preferably a *T. reesei cbh1* promoter or *A. niger* glucoamylase promoter.

Examples of preferred recombinant expression vector are the plasmids pALK945, pALK948, pALK1021 and pALK1022. The plasmid pALK1022 is shown in Figure 17. The constructs of the three other plasmids are essentially the same that of plasmid pALK1022 with the exception that the linker sequences are as those described in detail in the examples, e.g. SEQ ID NO: 11:, SEQ ID NO: 13:, SEQ ID NO: 15: and SEQ ID NO: 17:. Said expression vectors are used to transform filamentous fungi, such as *Aspergillus* and *Trichoderma*, most preferably *T. reesei*.

Hosts transformed with said expression vectors produce xylanases which all have the N-terminal amino acid sequence SEQ ID NO: 5:, which is identical with the N-terminal sequence (D-T-T-T-I-T-Q) of wild type *A. flexuosa* xylanase.

The transformed hosts are capable of expressing and secreting one or more proteins or enzymes, preferably xylanases or cellulases originating from bacteria or actinomycete into the culture medium during the cultivation or fermentation.

After the cultivation and removal of the host cells by filtration, centrifugation etc., the culture medium as such or concentrated provides a useful protein or enzyme preparation, which can be subjected to further down-stream processing methods for improved stability and storability. Alternatively, the preparation can be subjected to isolation and purification to obtain the desired protein or enzyme originating from bacteria or actinomycete in more purified form.

Examples of useful down-stream processes of the spent culture medium are e.g. filtration, ultrafiltration, precipitation, centrifugation, drying, evaporation, immobilization, granulation etc.

The preparation containing enzymes, especially xylanases and cellulases originating from actinomycetes and which are obtainable by cultivating hosts transformed with the expression vectors of the present invention are useful e.g. for enzyme-aided bleaching in because the enzymes are stable at processing temperatures, when the processing temperatures are in the ranges 50-90 °C, preferably 60-85 °C, most preferably 70-80 °C.

Said preparations are also useful for treating paper pulp and for enzymatical treating of plant biomass.

Thus, the present invention provides an alternative and improved method for producing enzyme containing preparation of bacterial, more preferably of actinomycetous origin in a filamentous fungal host by first constructing recombinant expression vectors, in which a promoter is operably linked to a DNA sequence of a filamentous fungus secretable protein or one or more functional domains of said protein, which in turn contain, fused in frame, a DNA sequence encoding a bacterial protein; then transforming a filamentous fungal host with the thus constructed vectors and cultivating said transformed hosts in a culture medium and under culture conditions, which are optimal for the secretion of the enzyme. After the cultivation the host cells are separated from the culture medium, which can be used as an

enzyme preparation as such or in concentrated form or after subjected to suitable down-stream processing methods.

The invention is described in more detail in the following examples. These examples show only a few concrete applications of the invention. It is self evident for one skilled in the art to create several similar applications. Hence the examples should not be interpreted to narrow the scope of the invention only to clarify the use of the invention.

Examples

Example 1
Actinomadura flexuosa ***DSM43186 Shake Flask and Fermentor Cultivations***

The strain *A. flexuosa* DSM43186 was streaked on rolled oats mineral medium plate (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH [German collection of microorganisms and cell cultures], *DSM Catalogue of strains*, 3rd ed., Braunschweig, Germany (1983); 1 liter contains 20 g agar, 20 g rolled oats, 1 ml trace element solution containing 100 mg $FeSO_4$ x 7 H_2O, 100 mg $MnCl_2$ x 4 H_2O, 100 mg $ZnSO_4$ x 7 H_2O / 100 ml; pH 9.0) and incubated at 50 °C until sporulating. A sporulating colony was inoculated in 10 ml of XPYB medium (Greiner-Mai, E. *et al.*, *System. Appl. Microbiol.* 9:97-109 (1987); Holtz, C. *et al.*, *Antonie van Leeuwenhoek* 59:1-7 (1991)); 1 liter contains 5 g oats spelt xylan, 5 g peptone from casein, 5 g yeast extract, 5 g beef extract, 0.74 g $CaCl_2$ x 2 H_2O; pH 9.0) and was incubated at 55 °C in a rotary shaker (250 rpm) for two to three days. An inoculum of 5 ml was then transferred to 250 ml of the same medium and incubated at the same conditions for three days. Xylanase activity obtained was 17 nkat/ml.

The procedure for two 1 L fermentations (Biostat M, B. Braun, Germany) was prepared as above. 10% (v/v) inoculum was used for the fermentations. The pH was maintained at pH 7.8 $\pm$ 0.2 by addition of ammonia (12.5% (v/v)) and phosphoric acid (17% (v/v)), the fermentation temperature was 50 °C. The fermentor was stirred at 400 rpm and the air flow was 1 L/min. Xylanase activities obtained were 32 and 58 nkat/ml (measured at pH 6.0 and 60 °C with 5 minutes incubation time). The culture media of the cultivations were used in protein purification and bleaching experiments.

Xylanase activities throughout the examples were measured according to Bailey, M.J. *et al.*, *J. Biotechnol.* 23:257-270 (1992) using 1% (w/v) birch xylan (Roth no. 7500) as a substrate. The assay conditions were, if not otherwise stated, pH 5.3 and 50 °C with an

incubation time of 5 minutes. One xylanase unit (1 nkat) is defined as the amount of enzyme that produces reducing carbohydrates having a reducing power corresponding to one nmole of xylose in one second from birch xylan under assay conditions. The International Unit (IU) is defined as the amount of enzyme that produces one micromole of measured end-product in one minute from the polymeric substrate, then 1 IU = 16.67 nkat.

Example 2

Determination of the pH and Temperature Dependencies of* Actinomadura flexuosa *Xylanase Activity from the Culture Medium

To determine the pH dependency for the *A. flexuosa* xylanase activity, samples of the culture medium from the shake flask cultivation (Example 1) were diluted in 50 mM McIlvains buffers (50 mM citric acid - 100 mM Na_2HPO_4) of pH-range 3.0 - 11.0. The final pH values of the enzyme buffer mixtures were 3.5, 4.5, 5.4, 6.4, 7.2, 8.0, 8.5, 9.7 and 11.2. Xylanase activity was measured at each pH at 50 °C, 5 min reaction. The xylanase activity exhibited 80-100% of its maximum activity in the pH range of about 5.4-8.0, showing maximum activity at about pH 6.4 (Figure 1).

For the thermal stability determination, samples from the culture supernatant were diluted in 50 mM McIlvain's buffers. Bovine serum albumin (BSA) was added to a concentration of 100 μg/ml and pepstatin A 10 μg/ml as well as phenyl methyl sulfonyl fluoride (PMSF) 174 μg/ml were added as protease inhibitors. The final pH values of the enzyme buffer mixtures were 6.9, 7.8 and 9.0. Samples were incubated in the absence of the substrate at 60 °C, 70 °C and 80 °C. Samples were taken at intervals of 0, 30, 60 and 120 minutes and immediately cooled on ice prior to the residual xylanase activity determination at 50 °C (5 min reaction in the corresponding pH). The enzyme was very stable when incubated at 60 °C and 70 °C; after 120 minutes incubation at 70 °C at pH 9 over 60% of xylanase activity was retained (Figure 2A, 2B and 2C).

Example 3

Purification of* Actinomadura flexuosa *Xylanases

Purification of xylanases from *A. flexuosa* growth medium was performed at +4 °C with chromatographic columns coupled to a FPLC apparatus (Pharmacia). Xylanase activity measurements were performed at 50 °C and at pH 6.5. Protein was monitored at 280 nm throughout the purification. Samples were run on polyacrylamide slab gels containing 0.1% SDS on a Bio-Rad Mini Protean II electrophoresis system and stained with Coomassie Brilliant Blue. A polyclonal antibody prepared against *Thermomonospora fusca* xylanase A

XynA, obtained from Prof. David Wilson, Cornell University, New York) was used to detect *A. flexuosa* xylanase(s) in Western blots. In the detection, Promega's ProtoBlot AP System was used.

A growth medium of the two 1 l fermentations described in Example 1 was pooled and centrifuged at 8,000 g for 30 min. The supernatant (1,500 ml) was diluted 1 + 2 with 12.5 mM Na_2HPO_4 pH 9 and adjusted to pH 8.6 with 1 M NaOH. This sample was applied, in two sets, on a DEAE Sepharose CL-6B (Pharmacia) ion-exchanger (2.5 x 29 cm) equilibrated with 12.5 mM Na_2HPO_4, pH 9, at 100 ml/h. The flow-through of both runs was combined and processed separately as described later.

Elution of the bound proteins from the DEAE-column (Figure 3) was accomplished by a linear gradient (400 ml + 400 ml) from 25 mM Na_2HPO_4 pH 9, to 25 mM Na_2HPO_4, pH 9 containing 1 M NaCl at a flow rate of 105 ml/h and fractions of 10 ml were collected. Two xylanase activity containing peaks could be collected (pool I and II), as well as a long "tailing" of the second peak (pool III).

The three pools (each combined from both DEAE runs) were adjusted to contain 2 M sodium chloride each and applied separately on a Phenyl Sepharose CL-4B (Pharmacia) column (2.5 x 15 cm) equilibrated with 25 mM Na_2HPO_4, pH 9 containing 2 M NaCl. Elution was performed at 100 ml/h with a two step gradient of 100% buffer A (25 mM Na_2HPO_4, pH 9) to 35% buffer B (25 mM Na_2HPO_4 containing 60% ethylene glycol) in 60 min followed by a steeper gradient from 35% B to 100% B in 60 min. Fractions of 7 ml (pool I) or 5 ml (pools II and III) were collected. The xylanase activity containing fractions of pool I obtained were pooled and named DEPS I (Figure 4A). Both DEAE pools II and III resulted in two xylanase activity containing peaks named DEPS II/1, DEPS II/2 (Figure 4B) and DEPS III/1, DEPS III/2 (Figure 4C), respectively.

Samples of these pools were dialyzed against 25 mM Na_2PO_4, pH 9, over night and run on SDS-PAGE and stained for protein with Coomassie Blue (Figure 5A) as well as analyzed by Western blots with *T. fusca* antibody (Figure 5B). The antibody reacted only with two to three bands of smaller molecular mass (35 kDa or lower) from the growth medium and weakly with the proteins in these pools. The apparent molecular masses of the proteins in these pools were 50 kDa as estimated from SDS-PAGE with molecular mass standards. Pools DEPS II/2, DEPS III/1 and DEPS III/2 were the most pure.

The flow-through of the DEAE ion-exchanger (see above) was concentrated with a cut-off membrane of 30 kDa. Roughly half of the xylanase activity was found in the concentrate

and half in the permeate. Both were adjusted to contain 2 M NaCl and applied on a Phenyl Sepharose 6 FastFlow (low sub; Pharmacia) column (2.5 x 34 cm) equilibrated with 25 mM Na_2HPO_4, pH 9, containing 2 M NaCl. Elution was accomplished at 300 mlh-1 with the same gradient as was used for DEAE pools on Phenyl Sepharose CL-6B and fractions of 10 ml were collected. Xylanase activity containing peaks obtained were named KF1, KF2 and KF3 (Figure 6B). The permeate from the concentration was subjected to an identical Phenyl Sepharose 6 FastFlow (low sub) run, and the xylanase activity containing fractions were named PF1 and PF2 (Figure 6A). These peaks were dialyzed over night against 25 mM Na_2HPO_4, pH 9, and analyzed on SDS-PAGE as well as on Western blots (Figure 7A and 7B). The first peak, KF_1, from the concentrate showed a band of 40 kDa apparent molecular mass on SDS-PAGE, but no reaction on Western blots. However, this peak had the highest xylanase activity. KF2 showed a band of 50 kDa on SDS-PAGE reacting weakly with the antibody, but a clear band of 30 kDa could be seen on Western blots. The third peak, KF3, showed a band of 35 kDa on Western blots. The concentrate contained xylanases with apparent molecular weights of 50, 40, 35 as well as 30 kDa. The first peak, PF1, from the permeate reacted with *T. fusca* antibody showing two bands of 35 kDa and 30 kDa, respectively. PF2, on the other hand, showed only one band of 35 kDa on Western blots.

As a summary, *A. flexuosa* sp. DSM43186 growth medium contains xylanases with molecular mass of about 50 kDa (represented by pools DEPSII/2.DEPSIII/1 and DEPSIII/2), 40 kDa (represented by pool KF1), 35 kDa (represented by pool PF2 and KF3) and 30 kDa (represented by pool KF2). Of these, the 35 kDa and 50 kDa proteins appear as the major xylanase bands (on SDS-PAGE). It is possible that the 40 kDa xylanase band on SDS-PAGE is a degradation product of the 50 kDa band on SDS-PAGE and that the 30 kDa band on SDS-PAGE is a degradation product of 35 kDa xylanase band on SDS-PAGE.

Example 4

Production and Sequencing of Peptides from the Purified 35 kDa and 50 kDa Xylanases

A sample (12 ml) of pool I from the DEAE Sepharose CL-6B (Fig. 3) run was subjected to gel exclusion chromatography on a HighLoad 26/60 Superdex G75 column (Pharmacia) equilibrated with 25 mM Na_2HPO_4, pH 9 at 120 ml/h. A sample (25 ml) of the xylanase activity containing peak fraction obtained was diluted (1 + 1) with water and applied on a mono Q (Pharmacia) ion-exchanger equilibrated with 12.5 mM Na_2HPO_4, pH 9. Elution was performed at 30 ml/h with a linear gradient from 12.5 mM Na_2HPO_4, pH 9 to 12.5 mM Na_2HPO_4, pH 9 containing 0.5 M NaCl in 50 min. The xylanase activity containing

peak (1 ml) was concentrated on a Centricon micro concentrator (cut-off 30 kDa) and eluted with 1% ammonium bicarbonate. This concentrated sample containing a single 50 kDa protein band on SDS-PAGE was evaporated and alkylated with vinylpyridin. The alkylated sample was digested with trypsin (modified trypsin, sequenal grade, Promega V5111). The digest was applied on a reverse phase column coupled to an HPLC, and peaks absorbing at 214 nm were collected manually. Each of the collected fractions were subjected to Edman degradation in a gas-pulsed-liquid-phase sequencer (Kalkkinen & Tilgmann, *J. Protein Chem.* 7:242-243 (1988)) and the released PTH amino acids were analyzed on-line by using narrow bore reverse phase HPLC.

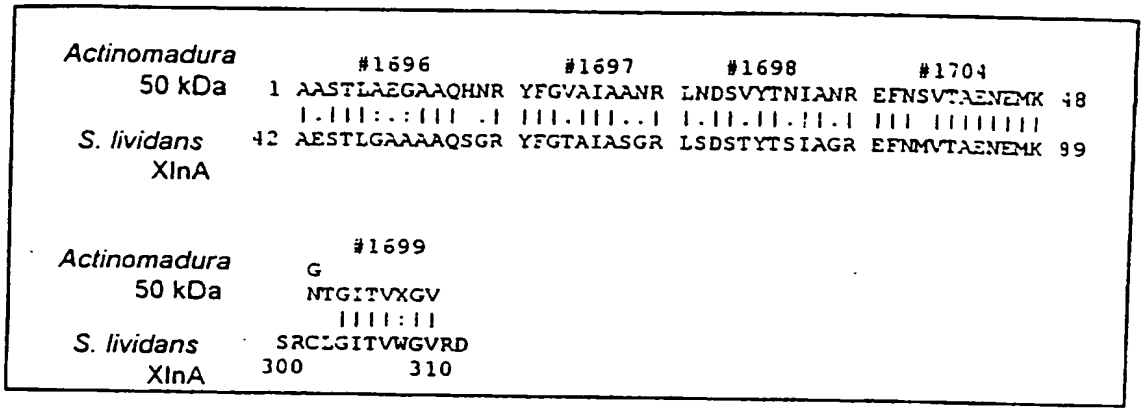
A sample of purified 35 kDa xylanase (purified essentially as described in Example 3) was subjected to Edman Degradation as above.

Peptides obtained from the purified 50 kDa xylanase and the N-terminal sequence of the purified 35 kDa xylanase are listed in Table 1.

Table 1: Peptides from the purified 50 kDa xylanase and the N-terminal sequence of the purified 35 kDa xylanase

Peptide	Sequence
# 1696	Ala-Ala-Ser-Thr-Leu-Ala-Glu-Gly-Ala-Ala-Gln-His-Asn-Arg
# 1697	Tyr-Phe-Gly-Val-Ala-Ile-Ala-Ala-Asn-Arg
# 1698	Leu-Asn-Asp-Ser-Val-Tyr-Thr-Asn-Ile-Ala-Asn-Arg
# 1699	Asn/Gly/X-Thr-Gly-Ile-Thr-Val-X-Gly-Val
# 1703	His/Glu/Thr-Glu/Phe-Leu/Asn-Val/Ser-Tyr/Val-Asn/Thr-Met /Ala-Val/ Glu-Asn/X-Glu/X-Met/X
# 1704	Glu-Phe-Asn-Ser-Val-Thr-Ala-Glu-Asn-Glu-Met-(Lys)
35 kDa N-term	Asp-Thr-Thr-Ile-Thr-Gln

The combination of the 50 kDa xylanase peptide sequences #1696 (SEQ ID NO: 6), #1697 (SEQ ID NO: 7), #1698 (SEQ ID NO: 8) and #1704 (SEQ ID NO: 10) corresponds with 75% similarity to amino acids 42-89 in *Streptomyces lividans* xylanase A (accession number M64551). The peptide #1703 has not been assigned any SEQ ID NO, because the multitude of alternatives present in the sequence and because it is not claimed. In addition, peptide #1699 (SEQ ID NO: 9:) shows 78% similarity to amino acids 301-309 in *S. lividans* XlnA:



The sequences of *S.lividans* are present for comparison only.

Example 5

The pH Properties and Temperature Stability of the Purified 35 kDa and 50 kDa Xylanases

The temperature stability of the purified 35 and 50 kDa enzymes with or without 100 μg/ml BSA was determined by incubating the enzyme samples at 70 °C, pH 6.0 for a period of 0, 2, 6 and 24 hours after which the xylanase activity of the samples was determined (at pH 6.5, 60 °C, 20 min reaction). In the samples into which BSA had been added, over 80% of the original activity could be measured even after 24 h of incubation (Figures 8 and 9 for the 35 kDa and the 50 kDa xylanases, respectively). When BSA was not added, still about 60% (35 kDa) or 70% (50 kDa) of the original activity was measured after 24 h of incubation (Figures 8 and 9).

The pH dependence was determined by incubating the enzyme samples at different pH values (pH 4-8) and at temperatures of 80 °C (35 kDa) and 60, 70 and 80 °C (50 kDa) for 20 minutes (35 kDa) or 10 minutes (50 kDa). At 80 °C, the 35 kDa xylanase showed a pH optimum of around pH 6 having nearly 90% of its activity from about pH 5 to 7 (Figure 10A). At 60 °C and 70 °C, the 50 kDa xylanase showed a pH optimum of pH 5-7 and at 80 °C, a pH optimum of pH 6-7. The enzyme was very stable from pH 5-7 under these conditions (Figure 10B). Incubation of both 35 kDa and 50 kDa xylanases at 60 °C for 60 minutes at pH values from 4.2 to 8.7 showed similar stability as found in the above experiment, except that the 50 kDa xylanase seems to be less stable at pH 4.2 under these

conditions (Figure 10C). Temperature dependence experiments at pH 7 with 60 minute incubations of the 35 kDa and 50 kDa xylanases with substrate at temperatures of 50, 60, 70 and 80 °C showed maximal activity at 70 °C for both enzymes (Figure 11). The 50 kDa xylanase seemed from these results to be slightly more stable at 80 °C and pH 7 than the 35 kDa xylanase. On the other hand, the 35 kDa xylanase showed more activity and stability in the pH range of 4-5 (Figures 10A-10C).

Example 6
Bleaching Experiments Using* Actinomadura flexuosa *Culture Medium

Bleaching experiments were done to determine the usefulness of *A. flexuosa* xylanase activity in both ECF (elementary chlorine free) and TCF (totally chlorine free) bleaching of kraft pulp.

ECF Bleaching

Growth medium containing *A. flexuosa* xylanase activity (Example 1) was added to Finnish oxygen delignified softwood kraft pulp (kappa number 15) in the amount of 50 or 100 nkat/g pulp dry matter. Xylanase activity was measured at pH 6 and 60 °C with 5 minutes incubation time. Cellulase activity of the growth medium was very low. The enzyme treatments were done at pH 7 and 70 °C for one hour. Reference pulp was kept under the same conditions without enzyme addition.

After the enzyme treatments pulps were bleached in two stages: chlorine dioxide stage and alkaline extraction. The absorbance of the filtrate at 280 nm was determined to estimate the amount of dissolved lignin.

As can be seen in Table 2, after the pretreatment with the xylanase preparation more residual lignin was removed from the pulps as evidenced by the increase of A_{280} of the filtrates and reduction of kappa numbers in the final pulps. The final pulps had also 3-4 units higher brightness compared with the reference. The strength of the pulps was not affected, because the viscosity values stayed inside the normal variation of the method.

Table 2

	Reference	50 nkat/g	100 nkat/g
Enzyme treatment			
Consistency, %	3	3	3
Retention time, hours	1	1	1
Enzyme dosage, nkat/g	0	50	100
Temperature, °C	70	70	70
pH, start/end	7.0/7.1	7.0/7.2	7.2/7.4
Absorbance, 280 nm	0.22	0.49	0.65
ClO_2 stage			
Consistency, %	3	3	3
Retention time, hours	1	1	1
ClO_2 dosage, %	2.3	2.3	2.3
Temperature, °C	60	60	60
pH at the end	2.4	2.5	2.5
Extraction stage			
Consistency, %	10	10	10
Retention time, hours	1	1	1
NaOH dosage, %	1.5	1.5	1.5
Temperature, °C	70	70	70
pH at the end	10.9	10.9	10.9
Final Pulp			
Brightness, % ISO	56.7	59.9	60.6
Kappa number	6.6	5.6	5.4
Viscosity, ml/g	920	910	900

TCF Bleaching

Finnish oxygen delignified softwood kraft pulp (kappa number 15) was treated with *A. flexuosa* xylanase preparation using enzyme dosages of 50 and 100 nkat/g pulp dry matter. Xylanase activity was measured at pH 6 and 60 °C with 5 minutes incubation time. Cellulase activity of the preparation was very low. The enzyme treatments were done at pH 7 and 70 °C for one hour. Reference pulp was kept under the same conditions without enzyme addition.

After the enzyme treatments the pulps were bleached using QP sequence. Metals were first removed by chelating with 0.2 % EDTA (chelating stage, Q) and the pulps were then bleached with hydrogen peroxide (peroxide stage, P). Bleaching chemicals were the following: 3 % H_2O_2, 3 % NaOH 0,2 % DPTA (diethylene triamine pentaacetic acid) and 0.5 % $MgSO_4$. The absorbance of the filtrate at 280 nm was determined to estimate the amount of dissolved lignin. The results are shown in Table 3.

Table 3

	Reference	50 nkat / g	100 nkat / g
Enzyme treatment			
Consistency, %	3.5	3.5	3.5
Retention time, hours	1	1	1
Enzyme dosage, nkat/g	0	50	100
Temperature, °C	70	70	70
pH, start/end	7.0/7.4	7.0/7.3	7.0/7.3
Absorbance, 280 nm	0.27	0.43	0.57
Chelation stage, Q			
Consistency, %	3.0	3.0	3.0
Retention time, hours	1	1	1
EDTA, % of dry matter	0.2	0.2	0.2
Temperature, °C	70	70	70
pH at the end	5.5	5.6	5.8
Absorbance, 280 nm	0.24	0.44	0.64
Peroxide stage, P			
Consistency, %	10	10	10
Retention time, hours	3	3	3
Temperature, °C	80	80	80
pH at the end	10.6	10.6	10.6
Peroxide dosage, %	3.0	3.0	3.0
Peroxide consumed, %	2.1	2.2	2.1
Final Pulp			
Brightness, %	71.9	72.9	73.0
Kappa number	9.0	8.3	7.9
Viscosity, ml/g	870	890	890

Table 3 shows that according to the measured A_{280} values and kappa numbers, significantly more lignin was removed after the xylanase treatments compared with the reference. The viscosity values of the pulps remained inside the normal variation of the method, which means that the strength of the pulps was not affected. Also brightness values were higher than reference, but as expected the increase was slighter than in chlorine dioxide bleaching.

Example 7

Bleaching Experiments Using the Purified 35 kDa and 50 kDa Xylanases

The purified larger 50 kDa (AM50) xylanase and the smaller 35 kDa (AM35) xylanase (including also the 30 kDa xylanase) were tested in a three stage peroxide bleaching. The purified enzyme preparations were the same as used in the determination of the pH and temperature properties of the purified enzymes (Example 5).

The purified enzyme preparations were added to Finnish oxygen delignified softwood kraft pulp (kappa number 13.5 and brightness 37 %) in the amount of 100 nkat/g pulp dry matter. Xylanase activity was measured at pH 6.5 and 60 °C with 5 minutes incubation time. The enzyme treatments were done at pH 6.5 and 60 °C for one hour. Reference pulp was treated in the same conditions but without enzyme addition. Bleaching was performed with $QP_1P_2P_3$ sequence. The chelation stage (Q) was performed by adding EDTA to 0.2 % of pulp dry matter. The three hydrogen peroxide stages ($P_1P_2P_3$) were all carried out the same way. The results are shown in Table 4.

Table 4

	Reference	AM50	AM35
Enzyme treatment			
Consistency, %	3.5	3.5	3.5
Retention time, hours	1	1	1
Enzyme dosage, nkat/g	0	100	100
Temperature, °C, start/end	60/59	58/58	60/59
pH, start/end	6.6/6.6	6.8/6.8	6.7/6.7

Chelation stage, Q			
Consistency, %	3.0	3.0	3.0
Retention time, hours	1	1	1
EDTA, % of dry matter	0.2	0.2	0.2
Temperature at the end, °C	50	49	51
pH at the end	4.6	5.4	4.8
P_1 stage			
Consistency, %	10	10	10
Retention time, hours	3	3	3
Temperature, °C	80	80	80
pH, start/end	12.0/11.7	12.1/12.0	11.9/11.7
Peroxide dosage, %	3.0	3.0	3.0
Peroxide consumed, %	2.7	2.7	2.6
Brightness, %	59.6	62.3	63.7
Kappa number	(5.9)	6.3	5.3
P_2 stage			
Consistency, %	10	10	10
Retention time, hours	3	3	3
Temperature, °C	80	80	80
pH, start/end	12.2/11.7	12.2/11.7	12.1/11.6
Peroxide dosage, %	3.0	3.0	3.0
Peroxide consumed, %	2.2	2.4	2.2
Brightness, %	67.2	69.7	70.7
Kappa number	6.8	4.8	4.9
P_3 stage			
Consistency, %	10	10	10
Retention time, hours	3	3	3
Temperature, °C	80	80	80
pH, start/end	11.9/12.0	12.0/11.6	12.0/11.8
Peroxide dosage, %	3.0	3.0	3.0
Peroxide consumed, %	2.1	2.2	2.0
Brightness, %	71.3	74.0	74.4
Kappa number	5.2	4.1	2.2
Total peroxide consumption, %	7.0	7.3	6.8

The use of AM50 and AM35 clearly increased the brightness without significantly increasing the amount of peroxide that was consumed. Also lignin content of pulps was reduced which is evidenced by the reduction of kappa numbers of pulps treated with these enzymes.

Example 8

Isolation of the Chromosomal DNA and Construction of the Genomic Library

Actinomadura flexuosa DSM43186 was cultivated in 50 ml of medium consisting of 10% (w/v) sucrose, 0.5% (w/v) oat spelt xylan, 0.5% (w/v) peptone from casein, 0.5% (w/v) yeast extract, 0.5% (w/v) beef extract, 0.074% (w/v) $CaCl_2$ x $2H_2O$, pH 7.4-7.5, in baffled shake flask for 2.5 days at 52 °C with shaking at 200 rpm. 2.5 ml of this culture was transferred to 50 ml of fresh medium supplemented with 0.8% glycine, and grown for 2 days at 50 °C, 200 rpm. Cells were pelleted by centrifugation and washed with 10% sucrose-25mM Tris-HCl (pH 8.0)-25mM EDTA.

The chromosomal DNA was isolated according to Hopwood *et al.*, Genetic manipulation of *Streptomyces*: A laboratory manual, The John Innes Foundation, Norwich, UK (1985). Briefly, the mycelium was lysed with lysozyme and 2 x Kirby mixture (2 g sodium triisopropylnaphthalene sulphonate, 12 g sodium 4-amino-salicylate, 5 ml 2 M Tris-HCl (pH 8.0), 6 ml of Tris-HCl saturated phenol, made up to 100 ml with water). The DNA was precipitated with isopropanol and dissolved into TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). RNA was digested with RNase.

The chromosomal DNA was partially digested with *Sau*3A (Boehringer, Germany) and size-fractionated in sucrose gradient (10-40% (w/v) sucrose in 1 M NaCl, 20 mM Tris-HCl, pH 8.0, 5 mM EDTA) run at 55 000 rpm for 6h at 22 °C in the Beckman TL-100 ultracentrifuge in the TLS-55 rotor. The gradient was divided in fractions, and those containing DNA of mainly 7-10 kb in size were used to construct a genomic *Actinomadura* library.

The predigested ZAP Express™ *Bam*HI/CIAP Vector Cloning Kit (Stratagene; U.S.A.) was used to construct the library and the instructions of the manufacturer were followed in all the subsequent steps. Briefly, about 200 ng of size-fractionated DNA was ligated into 1 μg of ZAP Express™ prepared arms, and packaged using Gigapack II packaging extract (Stratagene, U.S.A.). The titer of the library was determined by infecting *E. coli* XL1-Blue MRF cells with serial dilutions of the packaged phage and plating on NZY

plates. The total titer of the ligation mixture was approximately 3 x 10^7 pfu/ml, with over 96% insert frequency. The library was used for screening without amplification.

Example 9A

Isolation of the Gene Encoding the 35 kDa Xylanase on the Basis of Hydrolyzing Activity on RBB-Xylan Plates

The genomic library of *Actinomadura flexuosa* DSM43186 DNA in ZAP Express™ vector was screened for xylanolytic activity, as follows. The host, Stratagene *E. coli* XL-Blue MRF' cells were grown in LB + 0.2% (w/v) maltose + 10 mM $MgSO_4$ and adjusted to OD_{600}=0.5. The cells were infected with the recombinant library for 15 min at 37 °C and plated with NZY top agar on the NZY plates. The plates were incubated for 4 hrs at 42 °C, overlaid with nitrocellulose filters saturated with 10 mM IPTG to induce the *lac*Z-fusion protein expression, and incubated over night at room temperature.

The filters were washed with 50 mM K-phosphate buffer (pH 6.8), and transferred onto RBB-xylan + kanamycin (Km) plates. The plate has two layers; lower layer of 15 ml of regular LB + Km (40 μg/ml) and upper layer of 5 ml of RBB xylan (0.5% (w/v) RBB xylan, 1% (w/v) oats spelts xylan in LB + Km, buffered with 50 mM K-phosphate, pH 6.8). The plates were transferred to 50 °C for a second night to determine xylanolytic activity. Filters were removed, and the clear halo on the RBB-xylan + Km plates revealed the clones having xylanase activity. 22 positive plaques from the original NZY-plates were picked in SM buffer/chloroform.

The ZAP Express™ vector has been designed to allow simple, efficient *in vivo* excision and recircularization of any cloned insert contained within the lambda vector to form a phagemid containing the cloned insert. Briefly, the positive clones were incubated with XL1 Blue MRF cells with the ExAssist helper phage. After heat denaturation (70 °C, 15 min), and centrifugation, the excised phagemid pBK-CMV is packaged as filamentous phage particles in the supernatant. The rescued phagemid was mixed with XLOLR cells, and plated on LB/kanamycin (50 μg/ml) according to the manufacturer.

E. coli XLOLR cells transformed with the rescued phagemid DNAs were retested on RBB-xylan + Km. From the 22 originally positive clones 12 retained the xylanase activity. The phagemid DNAs were digested with *Eco*RI-*Pst*I, electrophoresed, blotted onto a nylon membrane, and hybridized with a digoxigenin-labeled 1.15 kb *T. fusca* xylanase fragment from pALK185 (Fig. 12). The plasmid pALK185 contains the *T. fusca xyn*A gene from pTX101 (Ghangas, G.S. *et al.*, *J. Bact.* *171*:2963-2969 (1994)). Four phagemids

hybridized with the *T. fusca* DNA probe, indicating that they carried gene(s) sharing some homology with the *T. fusca* fragment. These phagemids were designated pALK938, pALK939, pALK940 and pALK941. From the *A. flexuosa* DSM43186 chromosomal DNA, the *T. fusca xynA* probe hybridized to about a 4 kb *EcoRI-PstI* fragment.

Example 9B

Isolation of the Gene Encoding for the 35 kDa Xylanase on the Basis of Hybridizing to the* Thermomonospora fusca *xynA Gene

The genomic library of *Actinomadura flexuosa* DSM43186 DNA in ZAP Express vector was screened with a digoxigenin-labeled 1.15 kb *T. fusca* xylanase fragment from pALK185 (Fig. 12), according to supplier's instructions. 17 positive clones were picked. The phagemids were excised *in vivo*, as described above in example 9A. The *E. coli* clones harboring the positive phagemids were tested for xylanolytic activity on RBB-xylan, as described above in example 9A. 11 clones showed xylanolytic activity. One of the clones was chosen, and the plasmid was designated pALK1056.

Example 10

Isolation of the Gene Encoding the 35 kDa Xylanase on the Basis of Production of Polypeptide recognized by the* Thermomonospora fusca *XynA Antibody

The polyclonal antibody against *T. fusca* 32 kDa xylanase, XynA (See Example 3), was used to screen the *Actinomadura flexuosa* DSM 43186 genomic library. Stratagene XL1-Blue MRF' cells were grown in LB + 0.2% maltose + 10 mM $MgSO_4$ and diluted to OD_{600}=0.5. The cells were infected with the recombinant library for 15 min at 37 °C and plated with NZY top agar on the NZY plates. Plates were incubated for 3.5 hours at 42 °C, overlaid with nitrocellulose filters saturated with 10 mM IPTG, and incubated overnight at room temperature. Detection was performed with the 1:1500 diluted *T. fusca* XynA antibody using Promega's ProtoBlot AP System. Twelve positive clones, of which the clone 1.1 clearly gave the strongest signal, were picked in SM buffer/chloroform, and purified with a second round of screening.

The phagemids were excised *in vivo*, as described above in example 9A. The phagemids were then digested with *Eco*RI and *Pst*I, electrophoresed, blotted onto a nylon membrane and hybridized with a digoxigenin-labeled 1.15 kb *T. fusca* xylanase fragment from pALK185 (Fig. 12). Of the *A. flexuosa* DSM43186 chromosomal DNA, the *T. fusca xynA* probe hybridized to about a 4 kb *Eco*RI-*Pst* fragment. The clones were also tested for xylanolytic activity on RBB-xylan, as described above in example 9A. One clone (clone

1.1) was positive in both screens. The phagemid carried by this clone was designated pALK923.

Example 11

Restriction Enzyme Analysis and Sequencing of the Xylanase Gene Coding for the 35 kDa Protein

The plasmids pALK938 (DSM9399), pALK939 (DSM9900), pALK940 (DSM9901), pALK941 (DSM9902), pALK1056 (DSM9903) and pALK923 (DSM9322) were analyzed by restriction enzyme analysis, and were used for sequencing of the xylanase gene. The DNA was sequenced by using ABI (Applied Biosystems, U.S.A.) kits based on fluorescent-labeled T3 and T7 primers, or sequence-specific primers with fluorescent-labelled dideoxynucleotides, by the Taq dye primer cycle sequencing protocol in accordance with the supplier's instructions. Because of the high GC content in the *A. flexuosa* DNA, the sequencing reactions were performed with 10% (v/v) DMSO, at annealing temperature of 58-60 °C. Sequencing reactions were analyzed on ABI 373A sequencer, and the sequences obtained were characterized by using the Genetics Computer Group Sequence Analysis Software Package, version 7.2. The DNA sequence encoding the 35 kDa xylanase is presented in Figure 13. The sequence shows an ORF (open reading frame) of 1035 bp, predicting a polypeptide of 344 amino acids, and corresponding to a protein with a molecular weight of about 37.5 kDa. A putative signal processing site is found after alanine 43, and the predicted mature protein has a calculated molecular weight of about 32.9 kDa. The sequence data is thus in good agreement with the 35 kDa xylanase purification and sequencing results described in Examples 3 and 4. The 35 kDa gene sequence appeared identical in all the tested clones, except in the pALK923 DNA. pALK923 contained 93 bp of unknown sequence at the N-terminus of the insert, after which the *A. flexuosa* 35 kDa xylanase gene sequence started at the location corresponding to base pair 411 in Figure 13.

The sequence shows high homology towards xylanases from different organisms. At amino acid level, the gene shows about 76% identity towards the *T. fusca* XynA. In addition, the *A. flexuosa* 35 kDa xylanase shows 81 % identity with xylan binding domain of the *T. fusca* XynA (Irwing *et.al.*, *Appl. Env. Microbiol.60:* 763-770 (1995)). Thus, *A. flexuosa* 35 kDa xylanase contains a separate xylan binding domain separated from the catalytic domain by a linker region showing only approximately 40 % identity with the linker region of *T. fusca* XynA.

Example 12

Isolation of the 50 kDa Actinomadura flexuosa *Xylanase Gene*

The genomic library of *A. flexuosa* DSM43186 DNA in ZAP Express™ vector was screened using a DNA probe.

Oligonucleotide primers were designed based on the peptide sequences derived from the purified 50 kDa protein. The primer sequences are presented in Table 5. Because the combination of peptide sequences #1696 (SEQ ID NO: 6), #1697 (SEQ ID NO: 7), #1698 (SEQ ID NO: 8) and #1704 (SEQ ID NO: 10) corresponds with 75% similarity to amino acids 42-89 in *Streptomyces lividans* xylanase A, a 39 bp antisense oligo was synthesized, from bases 331 to 369 in the *S. lividans xlnA* sequence. The *S. lividans xlnA* 331-369as probe and the primers #1704 (SEQ ID NO: 10:, #1703as, #1696s were labeled with digoxigenin and terminal transferase, and used as probes in hybridization at 50 °C according to Boehringer, DIG DNA Labeling and Detection Nonradioactive, Applications Manual.

The #1704as and the *S. lividans xlnA* 331-339as probe recognized the same 1.0 kb *Eco*RI-*Pst*I fragment in *A. flexuosa* DNA. The fragment is different from the 4 kb fragment recognized by the *T. fusca xynA* probe (See Example 9A). Based on these results, the *S. lividans xlnA* 331-369as probe was used to screen the *A. flexuosa* library for the 50 kDa xylanase coding gene.

Three positive plaques were picked after an overnight detection. These clones were named Act.xyl.50/13, Act.xyl.50/14 and Act.xyl.50/15.

The phagemids containing the cloned *A. flexuosa* insert were excised as described in Example 9A. To determine the xylanase activity, the *E. coli* clones were streaked on RBB-xylan + Km plates as described in Example 9A, using the strain producing the *A. flexuosa* 35 kDa xylanase (from plasmid pALK923) as a positive control. The clones Act.xyl.50/13 and Act.xyl.50/14 showed xylanase activity, giving a clear halo around the colony.

Table 5: Oligonucleotide primers used in the detection of the gene coding for the *A. flexuosa* 50 kDa xylanase

Primer	DNA sequence
Actinomadura sp. DSM43186	
#1696s	GCA/C/G/TGCA/C/G/TCAA/G/CAC/TAAC/TA/CG
#1703as	ACCATA/GTTA/GTAA/C/G/TACA/C/G/TA
#1704as	TTCATC/TTCA/GTTC/TTCA/C/G/TGC
S. lividans xlnA 331-369as	CGTGAGTTCAACATGGTGACGGCCGAGAACGAGATGAAG
S. lividans xlnA 257-284s	AGAGCGGCCGCTACTTCGGCACCGCCAT
S. lividans xlnA 530-561as	CACGCCGTTGATGTGGTCGATCATCGCCTGGC

s = sense; as = antisense

Example 13

Sequencing the Gene for 50 kDa Actinomadura flexuosa *Xylanase Protein*

The phagemid DNAs from the Act.xyl.50/13 and Act.xyl.50/14 were named pALK927 and pALK928, respectively. The *S. lividans xlnA* 331-369as oligomer was used to sequence the *A. flexuosa* insert. In addition, two oligomers corresponding to nucleotides 257-284 and 530-561 in the *S. lividans xln*A sequence, as well as sequence-specific primers, were synthesized to obtain sequence from the cloned insert. The sequencing reactions were performed with 10% (v/v) DMSO, at the annealing temperature of 58 °C. The sequencing was performed as described in Example 11. The sequence of the 1864 bps of the *A. flexuosa* DSM43186 50 kDa xylanase gene is presented in Figure 14. Peptide sequences obtained from the purified 50 kDa protein are indicated by underlining of the derived amino acid sequence. The derived peptide sequence shows 70-71% identity towards *Actinomadura* sp. FC7 xylanase II (Figure 15A) and *S. lividans* xylanase A (Figure 15B) proteins. The sequence shows an ORF of 1479 bps, predicting a polypeptide of 492 amino acids, corresponding to a protein with a molecular weight of about 53.5 kDa.

Example 14

Production of Bacterial (Actinomycetous) Enzymes in Trichoderma reesei: *Production of* Thermomonospora fusca *Xylanase*

The expression cassette pALK193 (Fig. 16) was constructed for expression of the *T. fusca* xylanase gene, *xynA* (Ghangas *et al., J. Bacteriol. 171*:2963-2969 (1989): Irwin *et al., Appl. & Environ. Microbiol. 60*:763-770 (1994)) in *T. reesei*. In the expression cassette, the *T. fusca* xylanase gene is fused to the *T. reesei* cellobiohydrolase 1 (*cbh1*) signal sequence that is preceeded by the *cbh1* promoter. The 9.4 kb pALK193 expression cassette was cut from the vector backbone by *Eco*RI restriction. It was then isolated, purified and transformed into *T. reesei* ALKO2221 strain.

The expression fragment pALK193 contains:

* *T. reesei cbh1* promoter and signal sequence: The approximately 2.2 kb promoter sequence was derived from the plasmid pAMH110 (EP 244 234 - Fig. 15) and was originally isolated from *T. reesei* strain VTT-D-80133 (Teeri *et al., Bio/Technol. 1*: 696-699 (1983)). The sequence of the signal sequence and the promoter area preceeding the ATG was published by Shoemaker *et al., Bio/Technology 1*: 691-696 (1983)). In the *T. reesei* strain VTT-D-80133 the sequence preceeding the ATG is CCGCGGACTGCGCATC (a *Sac*II site is underlined, an additional cytosine in the DNA sequence, compared to the sequence by Shoemaker *et al. Bio/Technology 1:* 691-696 (1983) is bolded).

To make an exact fusion of the *T. fusca xynA* gene to the *cbh1* signal sequence, the 12 nucleotides after the *Sfi*I site in the *cbh1* signal sequence and the 5'-end of the *T. fusca* xylanase gene (to the internal *Mlu*I site, see Fig. 12 and 16) were synthesized by using polymerase chain reaction (PCR).

* *T. fusca* xylanase gene (*xynA*): The cloning of the *xynA* gene is published in Ghangas *et al., J. Bacteriol. 171*:2963-2969 (1989), and the sequence of the gene is published in Irwin *et al., Appl. & Environ. Microbiol. 60*:763-770 (1994). The sequence coding for the mature enzyme was fused (exact fusion) to the *cbh1* signal sequence. About a 0.7 kb *xynA* terminator region, to the *Sma*I site after the STOP codon of the *xynA* coding region, precedes the *cbh1* terminator fragment in the construction.

* *T. reesei cbh1* terminator: The 0.7 kb *Ava*II terminator fragment starting 113 bp before the STOP of the *cbh1* gene was added after the *T. fusca xynA* gene, to ensure termination of transcription. The terminator fragment derived from the plasmid pAMH110 (originally

isolated from *T. reesei* strain VTT-D-80133; Teeri *et al., Bio/Technol. 1*: 696-699 (1983)) and it contains three TAA codons in all reading frames preceding the terminator fragment (from *Nde*I site, see EP 244 234).

A. nidulans amdS gene: The gene has been isolated from *Aspergillus nidulans* VH1-TRSX6. It encodes acetamidase (Hynes *et al., Mol. Cell. Biol. 3*: 1430-1439 (1983)). Acetamidase enables the strain to grow by using acetamide as the only nitrogen source and this characteristic was used for selecting the transformants. The 3.1 kb fragment (*Spe*I - *Xba*I) from the plasmid p3SR2 (Kelly and Hynes, *EMBO J. 4*: 475-479 (1985)) is used in the plasmids. The fragment contains 1007 bps of the promoter area, 1897 bps of the coding region (introns included) and the 183 bps terminator area of the *amdS* gene.

T. reesei cbh1 3'-fragment:The fragment was isolated from *T. reesei* ALKO2466 by using plasmid rescue (1.7 kb, *Bam*HI - *Eco*RI, starting 1.4 kb after the gene's STOP codon. Suominen *et al.*, "High frequency one-step gene replacement in *Trichoderma reesei* II. Effects of deletions of individual cellulase genes," *Mol. Gen Genet. 241*: 523-530 (1993)). Strain ALKO2466 derives from the strain ALKO233 (Harkki *et al., Enzyme Microb. Technol. 13*: 227-233 (1991)). The 3'-fragment is used together with the promoter area (described above) to target the *T. fusca xynA* gene to the *cbh1* locus by homologous recombination.

Standard DNA methods were used for construction of the vectors (Sambrook *et al.*, "Molecular Cloning: A Laboratory Manual", 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989). The restriction enzymes, T4 DNA ligase, Klenow fragment of the DNA polymerase I, T4 DNA polymerase, polynucleotide kinase and *Taq* polymerase were from Boehringer (Germany) and New England Biolabs (U.S.A.). Each enzyme was used according to the supplier's instructions. Plasmid DNA was isolated by using Qiagen columns (Qiagen GmbH, Germany) or Promega Magic Minipreps (Promega, U.S.A.) according to the manufacturer's protocols. The oligonucleotides used in the PCR-reactions and in sequencing reactions were synthesized by a ABI (Applied Biosystems, U.S.A.) 381A DNA Synthesizer. DNA sequencing was done using ABI kits based on fluorescence-labelled primers, or when sequence-specific primers were used, on fluoresence-labelled dideoxynucleotides, by the *Taq* cycle sequencing method according to the supplier's instructions. Sequencing reactions were analyzed on an ABI 373A sequencer.

DNA fragments for cloning or transformations were isolated from low-melting- point agarose gels (FMC Bioproducts, U.S.A.) by freeze-thaw-phenol method (Benson, *Biotechniques 2*:66-58 1984) or by using Qiaex II Gel Extraction Kit (Qiagen GmbH,

Germany), the GeneClean^R or Mermaid Kits^{TM} (Bio 101 Inc., U.S.A.) according to the supplier's instructions.

T. reesei ALKO2221 was transformed with the isolated 9.4 kb *Eco*RI expression cassette of pALK193 (Fig. 16) as described by Penttilä *et al.*, *Gene 61*: 155-164 (1987)) with the modifications described in Karhunen *et al.*, *Mol. Gen. Genet 241*: 515-522 (1993). *T. reesei* transformants were transferred on a selective medium and purified through conidia. Transformants were stabilized by growing them on selective slants for two generations prior to sporulating on potato dextrose agar.

The culture supernatants of the transformants were analyzed by measuring the xylanase activity produced, by running samples on SDS-PAGE and by performing Western blots. Polyclonal antibody against *T. fusca* xylanase and purified *T. fusca* xylanase used in the Western blots were obtained from Prof. David Wilson (Cornell University, New York).

The xylanase activity from the culture supernatants was measured at 6.5, 60 °C and with 5 minutes incubation time. Prior to the assay the supernatants were incubated at 60 °C for 20 hours in 50 mM McIlvain's buffer with 200 μg/ml BSA to destroy *T. reesei*'s own xylanase activity. *T. fusca* supernatant's xylanase activity was unaffected by the 20 hours' incubation used prior to the reaction but the incubation used did destroy host's xylanase activity. The xylanase activity produced by the best pALK193 transformants was about 900 nkat/ml.

The *T. fusca* xylanase produced by the *T. reesei* transformants was not detectable on SDS-PAGE but was detected on the Western blots. The amount of the *T. fusca* xylanase produced by the transformants, based on the estimation from the Western blots where purified *T. fusca* xylanase was used as a control, was about 50-100 mg/l.

Example 15

***Production of Bacterial (Actinomycete) Enzymes in* Trichoderma reesei:**
Production of* Trichoderma reesei *ß-mannanase and* Actinomadura flexuosa *Xylanase Fusions

The *Trichoderma reesei* strains were constructed for *Actinomadura flexuosa* xylanase production (35 kDa xylanase, AM35). Strains overproduce *A. flexuosa* xylanase and are unable to produce *T. reesei's* endoglucanase II and cellobiohydrolase I. Such cellulolytic activity-deficient preparations, and the making of same by recombinant DNA methods, are

described in US 5,298,405 incorporated herein by reference or Suominen *et al.*, *Mol. Gen. Genet. 241*: 523-530 (1993). For the overproduction of *A. flexuosa* xylanase, the *am35* gene was fused to the *T. reesei* mannanase 1 gene's core/hinge region and the gene fusion was expressed from the strong *cbh1* promoter. Different protease cleavage sites were added between the mannanase and xylanase encoding sequences.

The plasmids pALK945, pALK948, pALK1021 and pALK1022 (Fig. 17) containing the sequences SEQ ID NO: 11:, SEQ ID NO: 13:, SEQ ID NO: 15 and SEQ ID NO: 17:, respectively. The plasmids were used in the construction of the *A. flexuosa* xylanase overproducing strains and are otherwise identical to each other, except that the fusion between the *man1* core/hinge and *am35* sequences differs (see Fig. 19, and below).

The plasmids pALK945, pALK948, pALK1021 and pALK1022 contain the following elements:

* *cbh1* (cellobiohydrolase 1) promoter: The promoter is from *Trichoderma reesei* VTT-D-80133 (Teeri *et al.*, *Bio/Technology 1*: 696-699 (1983)). The 2.2 kb *Eco*RI - *Sac*II fragment (Karhunen *et al.*, *Mol. Gen. Genet. 241*: 515-522 (1993)) is used in the constructs. The sequence preceeding the ATG was published by Shoemaker *et al.*, *Bio/Technology 1*:
691-696 (1983)). In the *T. reesei* strain VTT-D-80133 the sequence preceeding the ATG is CCGCGGACTGCGCATC (the *Sac*II site is underlined, an additional cytosine in the DNA sequence, compared to the sequence by Shoemaker et al. *Bio/Technology 1*: 691-696 (1983), is bolded).

To make an exact fusion, the 10 nucleotides of the promoter, from the *Sac*II site to the ATG, and the 5'-end of the *man1* gene (to the internal *Cla*I site, see Fig. 17) were synthesized by using polymerase chain reaction (PCR).

* the *man1* gene's core/hinge region: The *man1* gene codes for ß-mannanase that degrades mannans/glucomannans (Stålbrand *et al.*, *Appl. Environ. Microbiol. 61*: 1090-1097 (1995)). The gene has been isolated from *T. reesei* QM6a and its sequence is known (Stålbrand *et al.*, 1995). The 1.35 kb DNA fragment from nucleotides 1 to 1346 coding for the *man1* core/hinge region (amino acids from 1 to 379) was used in plasmids pALK945

and pALK948. The DNA fragment from nucleotides 1 to 1359 (amino acids 1 to 383) was used in plasmids pALK1021 and pALK1022. The *man1* core/hinge region was linked, from its C-terminal end, to the *am35* gene by using the PCR method to obtain four different fusions.

* the *am35* gene: The nucleotide sequence and deduced amino acid sequence of the am35 gene encoding a 35 kDa xylanase is presented in Fig. 13. The gene was cloned from a genomic library of *Actinomadura flexuosa* DSM43186 by using a plate activity assay (Example 9A). A 1.3 kb fragment from nucleotide 542 (the N-terminal Asp44) to the *Mlu*I site about 250 bps after the end of the gene (pALK1055, Fig. 18) was used in all plasmids. The gene was linked, from its N-terminal end, to the *man1* core/hinge sequence by using four different fusions.

* the *man1* core/hinge - am35 fusions: *man1* core/hinge was fused to the AM35 with or without a KEX-linker sequence, marked as ...KR..., representing ...Lys-Arg... in the list below. The fusion was done by PCR and the following amino acid sequences were formed (see Fig.19 for the DNA sequences):

man1 core/hinge + **synthetic sequence** + *am35 sequence*

pALK945	...PLYGR*DTT*...	= additional R
pALK948	...PLYG**RDKR***DTT*...	= KEX2-linker added
pALK1021	...PLYGQCGG*DTT*...	= no new amino acids
pALK1022	...PLYGQCGG**RDKR***DTT*...	= KEX2-linker added

A *Nru*I restriction site (TCGCGA) was introduced to pALK945 and pALK948 linkers to aid construction of the linkage between the two sequences. This was done by changing the native codon encoding glycine 379 in the *man1* core/hinge region (GGC) to a synthetic codon (GGT) and selecting the codon **CGC** for arginine. The N-terminal Asp44 of the am35 is encoded by **GAC**.

The fusion sequences were sequenced to ensure that no unwanted alterations had taken place.

The linker sequence used in the plasmid pALK945 has been used in the production of murine anti-2-phenyloxazolone IgG1 antibody from *T. reesei* as a fusion to the cellobiohydrolase I core/hinge region (WO 92/01797; Nyyssönen *et al.*, *Bio/Technology 11*: 591-595 (1993)). The fusions were cleaved at a low frequency by an extracellular, hitherto uncharacterisized *T. reesei* protease. The cleavage was made after the tyrosine residue in the CBHI linker region, two amino acids before the authentic N-terminus of the heavy chain Fd chain.

The linker sequences in the plasmids pALK948 and pALK1022 carried a synthetic spacer peptide, containing a KEX2-like protein processing signal, preceding the mature *Actinomadura* xylanase.

* the *cbh1* terminator: The 739 bp *Aval*I fragment (Karhunen *et al.*, *Mol. Gen. Genet. 241*: 515-522 (1993)) starting 113 bp before the STOP of the *cbh1* gene was added after the *am35* gene to ensure termination of transcription.

* the *amdS* gene: The fragment containing the amdS gene was the same as used in the construction of *T. fusca xynA* expression plasmid, see Example 14.

* the *cbh1* 3'-fragment: The fragment was the same as used in the construction of *T. fusca xynA* expression plasmid, see Example 14. The 3'-fragment is used together with the promoter area (described above) to target the *man1-am35* gene fusion to the *cbh1* locus by homologous recombination.

Standard DNA methods used in the construction of vectors pALK945, pALK948, pALK1021 and pALK1022 are described in Example 16. The 10.3 kb expression cassette was cut from the vector backbone by *Eco*RI restriction. The expression cassettes were then isolated, purified and transformed into ALKO3620 as described in Example 16, but other *Trichoderma* strains can be used as hosts as well.

In the host strain ALKO3620 the endoglucanase 2 (*egl2*) gene has been replaced by the 3.3 kb BglII - XbaI fragment from the plasmid pAN8-1 (Mattern *et al.*, *Fungal Genet. Newlett. 35*: 25 (1988)). This fragment contains a transformation marker gene, *ble* from *Streptoalloteichus hindustanus* (Drocourt *et al.*, *Nucl. Acids Res. 18*: 4009 (1990)). The *ble* gene confers resistance to several antibiotics, e.g. phleomycin and it is, in the construct, expressed from *Aspergillus nidulans gpdA* (glyseraldehyde-3-phosphate-dehydrogenase) promoter, *A. nidulans trpC* terminator is used to terminate the transcription. The

replacement was done by using the recombinant DNA methods described in US 5,298,405, incorporated herein by reference.

Example **16**

Characteristics of the* Actinomadura flexuosa *Xylanase Producing Transformants

Several purified *amdS* transformants were grown in shake flasks in a medium containing 4% whey, 1.5% complex nitrogen source derived from grain, 1.5% KH_2PO_4 and 0.5% $(NH_4)_2SO_4$. Cultures were maintained at 30 °C and 250 rpm for 7 days. *A. flexuosa* was cultivated in 1 l fermentor as described in Example 1.

The culture supernatants were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). CHBI was detected by Western blotting and immunostaining using a CBHI specific monoclonal antibody (CI-258 (Aho *et al.*, *Eur. J. Biochem. 200*: 643-649 (1991)) and the ProtoBlot Western blot AP system (Promega, U.S.A.) according to the recommendations of the manufacturer.

Some of the CBHI negative transformants were chosen for further characterization: e.g. the recombinant *T. reesei* strains ALKO3620/pALK945/8, ALKO3620/pALK945/6, ALKO3620/pALK948/27, ALKO3620/pALK1021/4 and ALKO3620/pALK1022/29 do not contain the *cbh1* gene. The *cbh1* gene is replaced by the *amdS* marker gene and the *man1-am35* fusion construct in pALK945, pALK948, pALK1021, pALK1022 expression cassettes. The *cbh1* gene replacement was verified in Southern hybridisations. The host strain ALKO3620 used in the transformations is deficient of the *egl2* gene (see Example 15) Thus, the strains do not produce *Trichoderma's* cellulase components EGII and CBHI.

Xylanase activities were measured at pH 5.3 and 50 °C, with an incubation time of 5 minutes in 50 mM Na_2HPO_4 buffer and pH 7 and 70 °C, with an incubation time of 5 minutes in 50 mM McIlvain's buffer, containing 100 µg/ml of BSA. The xylanase activity of one transformant per each transformation is presented in Table 6. The corresponding production levels of the transformants as estimated from the specific activity of purified *A. flexuosa* AM35 xylanase (18 000 BXU/mg at pH 7.0 and 70 °C) are shown in Table 6. Another shake flask cultivation was performed which contained two *cbh1* negative transformants obtained from the pALK945 transformation. Xylanase activities were measured at pH 7, 70 °C with an incubation time of 60 minutes. Results are presented in Table 6.

Table 6:
The xylanase activity and estimated production level of *T. reesei* transformants producing *A. flexuosa* 35 kDa xylanase.

xylanase	BXU/ml (pH 5.3,50°C, 5 min)	BXU/ml (pH 7, 70°C, 60 min)	BXU/ml pH 7, 70°C, 5 min)	AM35 g/l (estimate)
A. flexuosa culture medium	190	ND	440	0.02*
ALKO3620	3560	10	360	0
ALKO3620/ pALK945/6	ND	6230	ND	ND
ALKO3620 pALK945/8	10970	8350	14970	0.83
ALKO3620/ pALK948/27	6690	7010	11920	0.66
ALKO3620/ pALK1021/4	8400	6630	9800	0.54
ALKO3620/ pALK1022/29	7940	7410	14870	0.83

* Total xylanase activity in *A. flexuosa* culture medium.

The xylanase activity of the *T. reesei* host strain ALKO3620 is about ten times lower at optimum conditions for the AM35 protein (pH 7 and 70 °C) than it is under the conditions optimal for the *T. reesei* xylanase (pH 5.3, 50 °C). The lowest activity and production level was obtained with a transformant containing a fusion construct without any protease processing site (ALKO3620/pALK1021/4).

For the thermal stability determination, samples from the culture supernatants were diluted in 50mM McIlvain's buffers. BSA was added as a carrier protein to the concentration of 100 μg/ml. The xylanase activity was measured by incubating the enzyme samples at 70 °C, pH 7.0 for a period of 0, 15, 30 and 120 minutes after which the xylanase activity of the samples was determined at pH 7, 70 °C, using a 5 minutes reaction time. The results are shown in Figure 20. The thermal stability of the AM35 protein produced by

recombinant *T. reesei* strains transformed with the pALK945 and pALK1022 plasmids was identical with *A. flexuosa* xylanase. The AM35 protein produced by pALK948 and pALK1021 transformants was less stable (Figure 20).

Samples from the culture supernatants were run on polyacrylamide slab gels containing 0.1% SDS on Bio-Rad Mini Protean II electrophoresis system. A polyclonal antibody prepared against the purified ß-mannanase (pI 5.4) of *T. reesei* RutC30 (Stålbrand *et al.*, *Appl. Environ. Microbiol. 61*: 1090-1097 (1995)) was used to detect the mannanase in Western blots. In the detection, Promega's ProtoBlot AP System was used. The Western result is shown in Fig. 21. The molecular weight of the ß-mannanase protein in the culture medium of the host strain ALKO3620 (lane 5) and of all the transformants (lanes 6-10) is somewhat larger than that of the purified 53 kDa ß-mannanase protein sample (Stålbrand *et al.*, *J. Biotechnol. 29*: 229-242 (1993)), (lanes 2 and 3). In addition to the native ß-mannanase, the transformants ALKO3620/pALK945/8, ALKO3620/pALK945/6, ALKO3620/pALK948/27 and ALKO3620/pALK1022/29 (lanes 6-9) produce a smaller protein (about 50 kDa) reacts with the polyclonal mannanase antibody. This band represents the shortened mannanase protein obtained from the fusion constructs, and shows that the extracellular proteases have processed the fusion. In the strain ALK03620/pALK1021/4 (lane 10) two bands with molecular weights of about 70 and 80 kDa are obtained. These bands originate from the unprocessed fusion protein (MANI core/hinge + AM35 mature protein).

The culture supernatants were analysed also with a polyclonal antibody raised against the purified 35 kDA xylanase of *A.flexuosa* (Example 18). The result is presented in Figure 22. Transformants ALKO3620/pALK945/6, ALKO3620/pALK948/27 and ALKO3620/pALK10 22/29 showed one to two major bands on the Western blots. These bands were estimated to be 37-39 kDa and migrated parallel to the purified 35 kDa xylanase of *A. flexuosa*. In another transformant obtained from the transformation with pALK945 expression cassette the major band had a molecular weight of 31 kDa. Transformant ALKO3620/pALK1021/4 showed two protein bands of about 70-80 kDa which correlated with the molecular weight of the unprocessed fusion protein. The transformant also produced the processed form of the fusion protein.

When plain actinomycetes xylanase is expressed in *T. reesei* as a fusion of a homologous gene, high production levels of the heterologous protein can be achieved. When *Actinomadura am35* gene was expressed under the *cbh1* promoter as a mannanase fusion, the level of xylanase produced was about 500-800 mg/l (Example 14). When *T. fusca xynA* was expressed in *T. reesei* by linking it to the same promoter (*cbh1*), without a fusion to

homologous gene, only 50-100 mg/l of xylanase was produced (Example 16). Identity between the two actinomycetes xylanases, *Actinomadura* xylanase AM35 and *T. fusca* xylanase A is 76% at amino acid level.

Example 17

Bleaching Experiments Using the* Actinomadura flexuosa *Xylanase Synthesized in* Trichoderma reesei *as a Mannanase Fusion Protein

Bleaching experiments were done to determine the usefulness of the *A. flexuosa* AM35 xylanase activity synthesized in *Trichoderma reesei* as a mannanase fusion protein in TCF (totally chlorine free) bleaching of kraft pulp.

The culture media of transformants ALKO3620/pALK945/8, ALKO3620/pALK948/27 and ALKO3620/pALK1022/29 (Example 16) were added to Finnish oxygen delignified softwood kraft pulp (kappa number 16) in the amount of 100 nkat/g pulp dry matter. Xylanase activity was measured at pH 7 and 70 °C with 60 minutes incubation time. The enzyme treatments were done at pH 7 and 80 °C for one hour. Reference pulp was treated in the same way but without enzyme addition. Bleaching was performed using QP sequence. Metals were first removed by chelation with EDTA (chelation stage, Q) and the pulps were then bleached with hydrogen peroxide (peroxide stage, P). The results are shown in Table 7.

Table 7

	Reference	ALKO3620 pALK945/8	ALKO3620 pALK948/27	ALKO3620 pALK1022/29
Enzyme treatment				
Consistency, %	3.5	3.5	3.5	3.5
Retention time, hours	1	1	1	1
Enzyme dosage, nkat/g	0	100	100	100
Temperature, °C, start/end	81/79	82/80	80/77	83/79
pH, start/end	7.1/6.9	6.9/6.9	7.0/7.0	7.0/7.3
Chelation stage, Q				
Consistency, %	3.0	3.0	3.0	3.0
Retention time, hours	1	1	1	1
EDTA, % of dry matter	0.2	0.2	0.2	0.2
Temperature at the end, °C	75	76	74	76
pH at the end	5.2	5.1	5.0	4.9
Peroxide stage, P				
Consistency,	10	10	10	10
Retention time, hours	3	3	3	3
Temperature, °C	80	80	80	80
pH, start/end	11.3/10.8	11.4/10.8	11.4/10.8	11.4/10.9
Peroxide dosage, %	3.0	3.0	3.0	3.0
Peroxide consumed, %	2.3	2.3	2.3	2.3
Brightness, %	64.2	66.2	65.5	65.9
Viscosity, ml/g	840	850	850	870

The use of *A. flexuosa* xylanase activity containing culture media of transformants ALKO3620/pALK945/8, ALKO3620/pALK948/27 and ALKO3620/pALK1022/29 as a pretreatment of pulp in peroxide bleaching at 80 °C increased the brightness (2 units at its best) of the pulps obtained without increasing the amount of peroxide that was consumed. Viscosity of the pulps was not reduced because of low contaminating cellulase activity in the culture media.

Bleaching experiment was also performed at pH 7 and 70 °C for one hour using the same culture media as above. The brightness values of the final pulps were similar (1.2 units at its best) than when the enzyme pretreatments were performed at pH 7 and 80 °C.

Example 18

Purification and Characterization of the Recombinant* Actinomadura flexuosa *35 kDa Xylanase

A 0.5 mg sample of purified 35 kDa xylanase from *A. flexuosa* DSM43186 was sent to Diabor Ltd (Kiviharjuntie 11 A 4, FIN-90220 Oulu, Finland) in order to raise polyclonal antibodies in rabbits. The titer of the produced antibody was good, and a dilution of 1:10.000 was suitable for Western blots.

The protein products of the 35 kDa A. flexuosa DSM43186 xylanase gene in *T. reesei* of both ALKO3620/pALK945/8 and ALKO3620/pALK945/6 were identified by running samples of growth medium obtained from fermentor cultivations on SDS-PAGE followed by Western blotting with the wild-type 35 kDa xylanase antibody. Western blotting was performed with the ProtoBlot Western blot AP system (Promega, U.S.A) according to the manufacturer's instructions. Prestained Bio-Rad (LMW-standards) standard proteins were used as molecular mass standards.

The different bands identified from ALKO3620/pALK945/8 depended on growth conditions and growth time. As a whole, five different bands reacted with the 35 kDa xylanase antibody (Figure 23A). The upper band was estimated to be a 80 kDa protein. The four other band were estimated to be 27, 30, 31 and 34 kDa. *A. flexuosa* (wild type) 35 kDa xylanase was on the same gel estimated to be 39 kDa. Thus, the gene of ALKO3620/pALK945/8 did not produce a xylanase band of the same size as the wild-type xylanase. The mass of the upper band, 80 kDa, correlates well with the intact fusion protein. ALKO3620/pALK945/6 showed two to three bands on the western blots, also depending on growth conditions (Figure 23B). The upper band was estimated to about 80 kDa and thus probably the intact fusion protein. The main band on the blots of

ALKO3620/pALK945/6 was estimated to 39 kDa and migrated parallel to the wild-type 35 kDa xylanase. Growth medium samples after prolonged growth of the ALKO3620/pALK945/6 strain showed also a faint 31 kDa band on Western blots. The occurrence of smaller size xylanases after prolonged growth of the fungus suggests that post translational modifications, e.g. proteolysis, are present.

The samples of ALKO3620/pALK945/8 and ALKO3620/pALK945/6 above were rerun on SDS-PAGE and blotted on PVDF membrane. The bands obtained were cut out from the PVDF blot and subjected to Edman degradation as described in Example 4. All bands showed the same amino terminal sequence i.e. SEQ ID NO: 5: as the wild-type 35 kDa xylanase (D-T-T-I-T-Q-). Thus, C-terminal modifications must account for the observed differences in molecular masses.

In order to further characterize the gene products of the recombinant 35 kDa *A. flexuosa* xylanase, the different molecular mass xylanases were purified from suitable growth mediums of ALKO3620/pALK945/8 and ALKO3620/pALK945/6. Purification was essentially performed as described in Example 3. Samples of 40 to 150 ml growth medium, depending on the estimated xylanase content, were adjusted to pH 9.1 with 1 M NaOH. The samples were centrifuged at 11.000 g (+4 °C for 20 min). The supernatants were adjusted to 3.7 mS/cm and separately applied on a 5 x 18 cm DEAE-Sepharose FF (Pharmacia) column equilibrated with 20 mM Na_2HPO_4, pH 8.6, at a flow rate of 30 ml/min. Most of the *T. reesei* proteins bound to the DEAE, but the xylanase activity was found in the flow-through.

The flow-through fractions containing xylanase activity were pooled and NaCl was added to 2 M. The pool was applied on a 5 x 13 cm Phenyl-Sepharose FF (Pharmacia) equilibrated at a flow rate of 20 ml/min with 40 mM Na_2HPO_4 , pH 8.6, containing 2 M NaCl. Elution was performed at a flow rate of 20 ml/min with a gradient from 100% equilibration buffer to 100% 40 mM Na_2HPO_4,pH 8.6, in 20 min. The column was further washed with this buffer for 10 min. Elution was continued for 30 min with a gradient from 100 % 40 mM Na_2HPO_4, pH 8.6, to 100 % 40 mM Na_2HPO_4, pH 8.6, containing 60 % ethylene glycol. Fractions of 10 ml were collected and assayed for xylanase activity, on SDS-PAGE as well as on Western blots. Fractions containing purified xylanases were pooled and stored at -20 °C. Wild-type 35 kDa xylanase was purified as above from a 840 ml growth medium sample of *A. flexuosa* (DSM43186).

The purified recombinant *A. flexuosa* xylanases and the wild-type 35 kDa xylanase were run on SDS-PAGE (Figure 24). From ALKO3620/pALK945/6 a purified 39 kDa xylanase was

obtained and from ALKO3620/pALK945/8 a 30 kDa and a 27 kDa xylanase. These, as well as the wild-type 35 kDa xylanase, were subjected to mass spectrometric analysis on a Brucker Biflex Reflector MALDI-TODF (Brucker-Franzeen GmbH. Germany) mass spectrometer. The wild-type 35 kDa xylanase showed a mass of 32 857, in well agreement to the mass calculated from the *A. flexuosa* 35 kDa gene (32 876). Example 11. Thus, wild-type 35 kDa xylanase seems to be unglycosylated. The 39 kDa xylanase from ALKO3620/pALK945/6 gave a mass of 33 429 by mass spectrometry. Since the N-terminal of this sample was identical with the wild-type xylanase, probably either a C-terminal extension or glycosylation accounted for the 572 Da deference in mass. There are no stop codons in the gene sequence, following the wild-type stop codon, which could account for the larger mass of the recombinant xylanase. Thus, probably one or more of the five potential N-glycosylation sites (Asn-X-Ser/Thr, X=/Pro) have been glycosylated in *T. reesei*. The two 30 and 27 kDa xylanases purified from ALKO3620/pALK945/8 had the same N-terminal sequence as the wild-type 35 kDa xylanase. On the mass spectrometer their masses were determined to 23 974 and 21 974 respectively. The estimated molecular masses from SDS-PAGE were thus larger than the masses determined by mass spectrometry. The SDS-PAGE 39 kDa xylanase was renamed to 33.4 kDa and the 30 kDa xylanase to 23.8 kDa and the 27 kDa xylanase to 22 kDa. The wild-type *A. flexuosa* xylanase name was kept as 35 kDa xylanase.

The protein concentration of the purified xylanases was determined at A_{205} by the method of Scopes (Scopes, *Anal. biochem. 59:* 277-287, 1974). The K_m values and the k_{cat} values (based on protein concentration and molecular mass) of the purified xylanases were determined at pH 7 and 70 °C in the substrate range from 2 to 25 mg/ml (birch xylan, Roth 7 500). The k_{cat} values obtained were slightly smaller for both the 35 and 33.4 kDa xylanases as compared to the 22 and 23.8 kDa xylanases (Table 8). However, the K_m values for the 22 and 23.8 kDa xylanases (approximately 10 mg/ml) were 2.5 x higher than the corresponding K_m values of the 33.4 and 35 kDa xylanases (approximately 4 mg/ml). As judged from the protein sequence of the 35 kDa gene, the 22 and 23.8 kDa xylanase products miss the C-terminal xylan binding domain (see Example 11) resulting in less efficient binding to xylan. Roughly estimated the 22 kDa form contains only the core domain without the linker region. The 23.8 kDa form contains in addition part of the linker region.

Determination of the pI of the purified xylanases was performed by running samples on a 0.5 x 20 cm mono P chromatofocusing column (Pharmacia) equilibrated with 0.075 mM Tris-HCl pH 9.3. Elution was performed with polybuffer (10 ml polybuffer in 100 ml water and adjusted to pH 6 with 1 M acetic acid) at a flow rate of 0.5 ml/min and fractions of 0.5

ml were collected. The pH and xylanase activity of the fractions were determined, and the xylanase activity was found in the fraction corresponding to its pI (Table 8).

Table 8

Xylanase Strain (kDa)	mass (kDa)	Km (mg/ml)	kcat (1/s)	pI
33.4 ALKO3620/pALK945/6	33.429	3.8	1397	8.6
23.8 ALKO3620/pALK945/8	23.769	9.8	1525	7.6
22 ALKO3620/pALK945/8	21.974	10.0	1682	8.2
35 DSM43186	32.857	4.5	1135	8.5

The temperature and pH dependence of the purified xylanases were determined by incubating samples for 60 min with substrate in the pH range of 5.1 to 7.9 and at temperatures of 60, 70 and 80 °C, essentially as described in Example 5. From the results, it seems that the full length xylanases, 35 and 33.4 kDa, are slightly more active at higher temperatures and pH than the shorter, 22 and 23.8 kDa, xylanases (Figure 25).

The purified xylanases were tested for thermal stability by incubating samples at 80 °C and both pH 5 and pH 7 (in 50 mM McIlvains buffer). Samples were withdrawn at suitable time intervals, and the residual activity was measured at pH 7 and 70 °C (5 min incubation time). The half-lives of the xylanases are shown in Table 9. Both 22 and 23.8 kDa xylanases shoved a longer half-life than the full length 33.4 and 35 kDa xylanases under the conditions tested. This stability difference could be a result of the presence of the separate binding domain in the full length xylanases, destabilizing the structure at high temperatures in the absence of substrate. In the temperature dependence experiments (giving slightly different results), the presence of substrate binding to the xylan-binding domain may, on the contrary, stabilize the full length xylanases.

Table 9

Xylanase (kDa)	$t_{1/2}$ (min) (80°C, pH 5)	$t_{1/2}$ (min) (80°C, pH 7)
33.4	13	17
23.8	157	95
22	123	63
35	32	31

Apparently the truncations of the 35 kDa xylanase from the C-terminal end has some, but not severe. effects on the kinetic features of this xylanase. A 22 kDa fragment of the 35 kDa xylanase is still active and shows similar kinetics as its longer counterparts.

Example 19

***Bleaching Experiments Using Purified 22.0 kDa, 23.8 kDa and 33.4 kDa Forms of AM35 Xylanase from* Actinomadura flexuosa**

The bleach boosting effect of purified forms 22.0 kDa, 23.8 kDa and 33.4 kDa of AM35 xylanase (Example 18) was tested in one stage peroxide bleaching.

The purified forms were added to Finnish oxygen delignified softwood kraft pulp in the amount of 100 nkat / pulp dry matter as such without a protecting agent (results in Table 10) and with *T. reesei* culture medium suitable for protective background (results in Table 11). The xylanase activity of the purified forms as well as the *T. reesei* culture medium were measured at pH 7 70 °C with 5 minutes incubation time. The enzyme treatments were done at pH 8 80 °C for one hour. Reference pulps were treated in the same way but without enzyme addition. Bleachings were performed using QP sequence. Metal ions were first removed by adding EDTA 0.2 % of pulp dry matter (chelation stage, Q). The pulps were then bleached with hydrogen peroxide (P) using the following chemicals: 3 % H_2O_2, 3 % NaOH, 0.2 % DTPA and 0.5 % $MgSO_4$. The conditions of Q and P stages are shown in Tables 10 and 11.

Table 10

	Reference	22.0 kDa	23.8 kDa	33.4 kDa
Enzyme treatment				
Consistency, %	3.5	3.5	3.5	3.5
Retention time, hours	1	1	1	1
Enzyme dosage, nkat/g	0	100	100	100
Temperature, °C, start/end	80/79	80/79	79/79	81/80
pH, start/end	8.3/8.2	8.1/8.0	8.2/8.1	8.2/8.1
Chelation stage, Q				
Consistency, %	3.0	3.0	3.0	3.0
Retention time, hours	1	1	1	1
EDTA, % of dry matter	0.2	0.2	0.2	0.2
Temperature at the end, °C	73	73	72	74
pH at the end	5.8	5.6	5.7	5.8
Peroxide stage, P				
Consistency, %	10	10	10	10
Retention time, hours	3	3	3	3
Temperature, °C	80	80	80	80
pH, start/end	11.3/10.9	11.3/10.8	11.3/10.7	11.3/10.7
Peroxide dosage, %	3.0	3.0	3.0	3.0
Peroxide consumed, %	2.3	2.3	2.3	2.3
Brightness, %	64.7	65.0	65.6	65.5

The 33.4 kDa and 23.8 kDa forms of AM35 xylanase seemed to boost the bleaching effect in peroxide bleaching when pH was 8, temperature 80 °C, time one hour and enzyme dosage 100 nkat/g of dry pulp. The 22.0 kDa form did not enhance bleachability probably because of the missing substrate binding domain and incomplete linker region. Generally the increase of brightness was quite small, 0.9 units at its best. This might be due to the fact that the purified enzymes were added as such to 80 °C pulp without any protecting agent or carrier protein, such as *T. reesei* culture medium or BSA.

The second bleaching experiment was carried out the same way as the first one. Only the purified enzyme forms were first mixed with a *T. reesei* culture medium and then added to the pulps. This culture medium was similar to the culture medium from which the enzyme forms were purified. The results are shown in Table 11.

The result of the second bleaching experiment was similar to the first one. The treatments with the 33.4 kDa and 23.8 kDa forms increased brightness about 1.5 units compared with the treatment where only *T. reesei* culture medium was used. Also 22 kDa form gave about 1 unit brightness increase. The results show that the *T. reesei* culture medium worked as a protecting background and 33.4 kDa and 23.8 kDa protein forms, whose linker regions between catalytic and binding domains are intact, increased brightness values more than the 22 kDa form.

Table 11

	Reference	22.0 kDa	23.8 kDA	33.4 kDa	T.reesei culture medium used as a protection background
Enzyme treatment					
Consistency, %	3.5	3.5	3.5	3.5	3.5
Retention time, hours	1	1	1	1	1
Enzyme dosage, nkat/g	0	100 (+100*)	100 (+100*)	100 (+100*)	100*
Temperature, °C, start/end	81/80	80/80	80/80	82/82	81/80
pH, start/end	7.9/7.8	7.6/7.7	7.6/7.7	7.6/7.6	7.6/7.6
Chelation stage, Q					
Consistency, %	3.0	3.0	3.0	3.0	3.0
Retention time, hours	1	1	1	1	1
EDTA, % of dry matter	0.2	0.2	0.2	0.2	0.2
Temperature at the end, °C	76	75	75	76	77
pH at the end	5.2	5.2	5.2	5.2	5.1
Peroxide stage, P					
Consistency, %	10	10	10	10	10
Retention time, hours	3	3	3	3	3
Temperature, °C	80	80	80	80	80
pH, start/end	11.4/ 10.7	11.4/ 10.7	11.5/ 10.8	11.5/ 10.8	11.6/ 10.8
Peroxide dosage, %	3.0	3.0	3.0	3.0	3.0
Peroxide consumed, %	2.2	2.3	2.3	2.3	2.3
Brightness, %	62.1	64.1	64.6	64.5	63.0

*) The xylanase activity is originated in the *T. reesei* culture medium consisting *T. reesei*'s own xylanases.

Example 20

***Production of 23.8 kDa form of* Actinomadura flexuosa *35 kDa xylanase in* Trichoderma reesei**

T. reesei strains are constructed for the production of xylanolytically active but shortened, still catalytically active fragments of the full-length actinomycete enzymes. Especially, *T. reesei* strains for the production of the 23.8 kDa form (see example 18) of *A. flexuosa* 35 kDa xylanase are constructed. The expression of the shortened form can lead to enhanced xylanase activity in the culture medium.

A recombinant vector encoding a xylanase or a desired domain of it is prepared by fusing the sequence encoding xylanase or a desired domain of it with the sequence of a secretable fungal protein or one or more functional domains of said protein. Especially, the sequence encoding the 23.8 kDa form of the *A. flexuosa* 35 kDa xylanase is fused to *T. reesei* cellulase or hemicellulase as described in US 5,298,405, WO 93/24621 and Stålbrand *et al.*, *Appl.Environ.Microbiol. 61*: 1090-1097 (1995) incorporated herein by reference. Especially, the enzyme is selected from the group consisting of CBHI, CBHII, EGI, EGII, XYLI, XYLII and MANI, or a functional domain thereof.

Fusion proteins can be constructed that contain an N-terminal mannanase, xylanase, cellobiohydrolase or endoglucanase core domain or the core and the hinge domains from the same, fused to the *A. flexuosa* xylanase sequence encoding the 23.8 kDa form. The result is a protein that contains N-terminal mannanase, xylanase, cellobiohydrolase or endoglucanase core or core and hinge regions and a C-terminal *A. flexuosa* 23.8 kDa form. The fusion protein contains both the mannanase, xylanase, cellobiohydrolase or endoglucanase and xylanase activities of various domains as provided in the fusion construct.

Fusion proteins can also be constructed such that the mannanase or cellobiohydrolase or endoglucanase tail or a desired fragment thereof, is included, placed before the *A. flexuosa* xylanase sequence, especially so as to allow use of a nonspecific protease site in the tail as a protease site for the recovery of the cellulase sequence from the expressed fusion protein. Alternatively, fusion proteins can be constructed that provide for a protease site in a linker that is placed before the *A. flexuosa* xylanase cellulase, with or without tail sequences.

Example 21

***Production of* Thermomonospora fusca *Cellulases in* Trichoderma reesei**

T. fusca produces at least six cellulase degrading enzymes, four endoglucanases E1, E2, E4 and E5 and two exocellulases E3 and E6 (Irwin *et al.*, *Biotechnol. and Bioeng. 42*: 1002-1013 (1993)). *T. reesei* strains are constructed for efficient production of *T. fusca* cellulases. Especially, *T. reesei* strains are constructed that express *T. fusca* endocellulase, E5. The cloning of the E5 gene is described in Lao *et al.*, . *Bacteriol. 173*: 3397-3407 (1991).

A recombinant vector encoding a cellulase is prepared by fusing the sequence encoding a polypeptide with cellolytic activity with the sequence of a secretable fungal protein or at least a functional domain of said protein. Especially the *T. fusca* cellulase encoding sequence is fused to *T. reesei* cellulase or hemicellulase or one or more functional domains of said cellulase or hemicellulase, as described in US 5,298,405, WO 93/24621 and Stålbrand *et al.*, *Appl. Environ. Microbiol.* 61: 1090-1097 (1995)) incorporated herein by reference. Especially, the enzyme is selected from the group consisting of CBHI, CBHII, EGI, EGII, XYLI, XYLII and MANI, or a domain thereof, such as secretion signal or the core sequence.

Fusion proteins can be constructed that contain an N-terminal mannanase, xylanase, cellobiohydrolase or endoglucanase core domain or the core and the hinge domains from the same, fused to the *T. fusca* cellulase sequence. The result is a protein that contains N-terminal mannanase, xylanase, cellobiohydrolase or endoglucanase core or core and hinge regions and a C-terminal *T. fusca* cellulase. The fusion protein contains both the mannanase, xylanase, cellobiohydrolase or endoglucanase and cellulase activities of various domains as provided in the fusion construct.

Fusion proteins can also be constructed such that the mannanase or cellobiohydrolase or endoglucanase tail or a desired fragment thereof, is included, placed before the *T. fusca* cellulase sequence, especially so as to allow use of a nonspecific protease site in the tail as a protease site for the recovery of the cellulase sequence from the expressed fusion protein. Alternatively, fusion proteins can be constructed that provide for a protease site in a linker that is placed before the *T. fusca* cellulase, with or without tail sequences.

Example 22

Bacterial Protein Expressed in Aspergillus

A recombinant vector encoding a bacterial enzyme is prepared by fusing the enzyme encoding sequence to an *Aspergillus* secretable protein. The protein is preferably *A. niger* or *A. niger* var *awamori* glucoamylase or α-amylase; or one or more functional domains

thereof. (Stoffer *et al., Biochem. J. 292*:197-202 (1993); Svensson *et al.*, Structure-Function relationship in amylases, Ed. R.B. Friedman. *Biotechnology of Amylodextrin Oligosaccharides. ACS Symposium Serium 458*:28-43 (1991); Boel *et al., EMBO J.* 3:1581-1585 (1984), Boel *et al., EMBO J. 3*: 1097-1102 (1984). Korman *et al. Curr. Genet 17*: 203-212, (1990)

Fusion proteins can be constructed that contain an N-terminal glucoamylase or α-amylase or one or more functional domains or from the same, fused to a sequence encoding bacterial protein such as *Actinomadura* xylanase. The result is a protein that contains N-terminal glucoamylase or α-amylase or part of them, and a C-terminal *Actinomadura* xylanase. The fusion protein contains both the mannanase or glucoamylase or α-amylase and xylanase activities of the various domains as provided in the fusion construct.

Fusion proteins can also be constructed such that e.g. glucoamylase tail or a desired fragment thereof, is included, placed before the *Actinomadura* xylanase sequence, especially so as to allow use of a nonspecific protease site in the tail as a protease site for the recovery of the xylanase sequence from the expressed fusion protein. Alternatively, fusion proteins can be constructed that provide for a protease site in a linker that is placed before the *Actinomadura* xylanase, with or without tail sequences.

The expression vector uses e.g. *A. niger* glucoamylase promoter. (Boel *et al., EMBO J. 3*:1581-1585 (1984)). The transformation host may be some *Aspergillus niger* strain (Kelly and Hynes, *EMBO J. 4*:475-479 (1985)) or for example some *Aspergillus niger var awamori* strain (e.g. ATCC 38854). The chosen *Aspergillus* strain is transformed similar to that described by Kelly and Hynes, *EMBO J. 4*:475-479 (1985)).

The *Actinomadura* xylanase producing transformants are then characterized similar to Example 16 with modification obvious to a person skilled in the art. The culture medium used may be *Aspergillus* complete medium. (Rowlands *et al., Mol. Gen. Genet. 126:201-216* (1973)).

All references cited herein are incorporated herein by reference. While this invention has been described in detail and with reference to specific embodiments thereof, it will be apparent to one skilled in the art that various changes and modifications could be made therein without departing from the spirit and scope thereof.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Primalco Ltd
(B) STREET: Valta-akseli
(C) CITY: Nurmijarvi
(E) COUNTRY: Finland
(F) POSTAL CODE (ZIP): FIN-05200
(G) TELEPHONE: +358 9 133 11
(H) TELEFAX: +358 9 133 1546

(ii) TITLE OF INVENTION: Production and Secretion of Proteins of Bacterial Origin in Filamentous Fungi

(iii) NUMBER OF SEQUENCES: 18

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1375 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:303..1337
(D) OTHER INFORMATION:/product= "AM35 xylanase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCCGGGTATT CATGTGAATG ATTAGCAACA GTTATGTTAC GGAGATATTT CTGAGAGTGT 60

TGACAGGTCG TGAAGTCGGT CCGATACTTT CGAGCTAGCT CCGATAGTTT TCGATACGCC 120

GGCACATCGA GCACGTCGGA CGAGTCACGC GCCACGTCGG TTTTCCGCCG CACGCCGCGC 180

```
AGAGCGGCCG GAGAACCCCC GCGTGTCCGC GGCATCGGTG CCGGTCCGTC GTTCGCCGCC      240

GACCGCGCGC CGGGTCGCGA CACGCCAGCC CCCATCGGCC CTTCTTCACG AGGAAGCCGT      300

AC ATG AAC GAA CCC CTC ACC ATC ACG CAG GCC AGG CGC CGC AGA CGC          347
   Met Asn Glu Pro Leu Thr Ile Thr Gln Ala Arg Arg Arg Arg Arg
     1               5                  10                  15

CTC GGC CTC CGG CGC ATC GTC ACC AGT GCC TTC GCC CTG GCA CTC GCC        395
Leu Gly Leu Arg Arg Ile Val Thr Ser Ala Phe Ala Leu Ala Leu Ala
                 20                  25                  30

ATC GCC GGT GCG CTG CTG CCC GGC ACG GCC CAC GCC GAC ACC ACC ATC        443
Ile Ala Gly Ala Leu Leu Pro Gly Thr Ala His Ala Asp Thr Thr Ile
             35                  40                  45

ACC CAG AAC CAG ACC GGG TAC GAC AAC GGC TAC TTC TAC TCG TTC TGG        491
Thr Gln Asn Gln Thr Gly Tyr Asp Asn Gly Tyr Phe Tyr Ser Phe Trp
         50                  55                  60

ACC GAC GCG CCC GGG ACC GTC TCC ATG ACC CTC CAC TCG GGC GGC AGC        539
Thr Asp Ala Pro Gly Thr Val Ser Met Thr Leu His Ser Gly Gly Ser
     65                  70                  75

TAC AGC ACC TCG TGG CGG AAC ACC GGG AAC TTC GTC GCC GGC AAG GGC        587
Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly Lys Gly
 80                  85                  90                  95

TGG TCC ACC GGG GGA CGG CGG ACC GTG ACC TAC AAC GCC TCC TTC AAC        635
Trp Ser Thr Gly Gly Arg Arg Thr Val Thr Tyr Asn Ala Ser Phe Asn
                100                 105                 110

CCG TCG GGT AAC GGC TAC CTC ACG CTC TAC GGC TGG ACC AGG AAC CCG        683
Pro Ser Gly Asn Gly Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Asn Pro
            115                 120                 125

CTC GTC GAG TAC TAC ATC GTC GAG AGC TGG GGC ACC TAC CGG CCC ACC        731
Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg Pro Thr
        130                 135                 140

GGC ACC TAC AAG GGC ACC GTC ACC ACC GAC GGG GGA ACG TAC GAC ATC        779
Gly Thr Tyr Lys Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr Asp Ile
    145                 150                 155

TAC GAG ACC TGG CGG TAC AAC GCG CCG TCC ATC GAG GGC ACC CGG ACC        827
Tyr Glu Thr Trp Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr Arg Thr
160                 165                 170                 175

TTC CAG CAG TTC TGG AGC GTC CGG CAG CAG AAG CGG ACC AGC GGC ACC        875
Phe Gln Gln Phe Trp Ser Val Arg Gln Gln Lys Arg Thr Ser Gly Thr
                180                 185                 190
```

```
ATC ACC ATC GGC AAC CAC TTC GAC GCC TGG GCC CGC GCC GGC ATG AAC        923
Ile Thr Ile Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly Met Asn
            195                 200                 205

CTG GGC AGC CAC GAC TAC CAG ATC ATG GCG ACC GAG GGC TAC CAG AGC        971
Leu Gly Ser His Asp Tyr Gln Ile Met Ala Thr Glu Gly Tyr Gln Ser
        210                 215                 220

AGC GGT AGC TCC ACC GTC TCC ATC AGC GAG GGT GGC AAC CCC GGC AAC       1019
Ser Gly Ser Ser Thr Val Ser Ile Ser Glu Gly Gly Asn Pro Gly Asn
    225                 230                 235

CCG GGT AAC CCC GGC AAC CCC GGC AAC CCC GGT AAC CCG GGT AAC CCC       1067
Pro Gly Asn Pro Gly Asn Pro Gly Asn Pro Gly Asn Pro Gly Asn Pro
240                 245                 250                 255

GGC GGT GGC TGC GTC GCG ACC CTC TCC GCC GGC CAG CAG TGG AGC GAC       1115
Gly Gly Gly Cys Val Ala Thr Leu Ser Ala Gly Gln Gln Trp Ser Asp
                260                 265                 270

CGC TAC AAC CTC AAC GTC TCG GTC AGC GGC TCG AAC AAC TGG ACG GTC       1163
Arg Tyr Asn Leu Asn Val Ser Val Ser Gly Ser Asn Asn Trp Thr Val
            275                 280                 285

CGG ATG GAC GTG CCC TAC CCG GCC CGC ATC ATC GCC ACC TGG AAC ATC       1211
Arg Met Asp Val Pro Tyr Pro Ala Arg Ile Ile Ala Thr Trp Asn Ile
        290                 295                 300

CAC GCC CAG TGG CCC GAG TCC CAG GTG CTC ATC GCC AGA CCC AAC GGC       1259
His Ala Gln Trp Pro Glu Ser Gln Val Leu Ile Ala Arg Pro Asn Gly
    305                 310                 315

AAC GGC AAC AAC TGG GGC GTG ACG ATC CAG CAC AAC GGC AAC TGG ACC       1307
Asn Gly Asn Asn Trp Gly Val Thr Ile Gln His Asn Gly Asn Trp Thr
320                 325                 330                 335

TGG CCG ACG GTC ACC TGT ACC GCG AAC TGA GTTCCCGCCC CCAAAGGTGG         1357
Trp Pro Thr Val Thr Cys Thr Ala Asn  *
                340                 345

CGCGGCGGCT CCCGGCCG                                                  1375
```

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 345 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Asn Glu Pro Leu Thr Ile Thr Gln Ala Arg Arg Arg Arg Arg Leu
 1               5                  10                  15
```

Gly Leu Arg Arg Ile Val Thr Ser Ala Phe Ala Leu Ala Leu Ala Ile
20 25 30

Ala Gly Ala Leu Leu Pro Gly Thr Ala His Ala Asp Thr Thr Ile Thr
35 40 45

Gln Asn Gln Thr Gly Tyr Asp Asn Gly Tyr Phe Tyr Ser Phe Trp Thr
50 55 60

Asp Ala Pro Gly Thr Val Ser Met Thr Leu His Ser Gly Gly Ser Tyr
65 70 75 80

Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly Lys Gly Trp
85 90 95

Ser Thr Gly Gly Arg Arg Thr Val Thr Tyr Asn Ala Ser Phe Asn Pro
100 105 110

Ser Gly Asn Gly Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Asn Pro Leu
115 120 125

Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg Pro Thr Gly
130 135 140

Thr Tyr Lys Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr Asp Ile Tyr
145 150 155 160

Glu Thr Trp Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr Arg Thr Phe
165 170 175

Gln Gln Phe Trp Ser Val Arg Gln Gln Lys Arg Thr Ser Gly Thr Ile
180 185 190

Thr Ile Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly Met Asn Leu
195 200 205

Gly Ser His Asp Tyr Gln Ile Met Ala Thr Glu Gly Tyr Gln Ser Ser
210 215 220

Gly Ser Ser Thr Val Ser Ile Ser Glu Gly Gly Asn Pro Gly Asn Pro
225 230 235 240

Gly Asn Pro Gly Asn Pro Gly Asn Pro Gly Asn Pro Gly Asn Pro Gly
245 250 255

Gly Gly Cys Val Ala Thr Leu Ser Ala Gly Gln Gln Trp Ser Asp Arg
260 265 270

Tyr Asn Leu Asn Val Ser Val Ser Gly Ser Asn Asn Trp Thr Val Arg
275 280 285

Met Asp Val Pro Tyr Pro Ala Arg Ile Ile Ala Thr Trp Asn Ile His
290 295 300

```
Ala Gln Trp Pro Glu Ser Gln Val Leu Ile Ala Arg Pro Asn Gly Asn
305                 310                 315                 320

Gly Asn Asn Trp Gly Val Thr Ile Gln His Asn Gly Asn Trp Thr Trp
                325                 330                 335

Pro Thr Val Thr Cys Thr Ala Asn  *
            340                 345
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1864 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:194..1672
(D) OTHER INFORMATION:/product= "AM50 xylanase"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
TTCGGCAGCC TATTGACAAA TTTCGTGAAT GTTTCCCACA CTTGCTCTGC AGACGGCCCC      60

GCCGATCATG GGTGCACCGG TCGGCGGGAC CGTGCTCCGA CGCCATTCGG GGGTGTGCGC     120

CTGCGGGCGC GGCGTCGATC CCGCGGGGAC TCCCGCGGTT CCCTTTCCGT GTCCCTCTAA     180

TGGAGGCTCA GGC ATG GGC GTG AAC GCC TTC CCC AGA CCC GGA GCT CGG          229
               Met Gly Val Asn Ala Phe Pro Arg Pro Gly Ala Arg
                                   350                 355

CGG TTC ACC GGC GGG CTG TAC CGG GCC CTG GCC GCG GCC ACG GTG AGC        277
Arg Phe Thr Gly Gly Leu Tyr Arg Ala Leu Ala Ala Ala Thr Val Ser
        360                 365                 370

GTG GTC GGC GTG GTC ACG GCC CTG ACG GTG ACC CAG CCC GCC AGC GCC        325
Val Val Gly Val Val Thr Ala Leu Thr Val Thr Gln Pro Ala Ser Ala
    375                 380                 385

GCG GCG AGC ACG CTC GCC GAG GGT GCC GCG CAG CAC AAC CGG TAC TTC        373
Ala Ala Ser Thr Leu Ala Glu Gly Ala Ala Gln His Asn Arg Tyr Phe
390                 395                 400                 405
```

```
GGC GTG GCC ATC GCC GCG AAC AGG CTC ACC GAC TCG GTC TAC ACC AAC          421
Gly Val Ala Ile Ala Ala Asn Arg Leu Thr Asp Ser Val Tyr Thr Asn
                410                 415                 420

ATC GCG AAC CGC GAG TTC AAC TCG GTG ACG GCC GAG AAC GAG ATG AAG          469
Ile Ala Asn Arg Glu Phe Asn Ser Val Thr Ala Glu Asn Glu Met Lys
            425                 430                 435

ATC GAC GCC ACC GAG CCG CAG CAG GGG CGG TTC GAC TTC ACC CAG GCC          517
Ile Asp Ala Thr Glu Pro Gln Gln Gly Arg Phe Asp Phe Thr Gln Ala
        440                 445                 450

GAC CGG ATC TAC AAC TGG GCG CGC CAG AAC GGC AAG CAG GTC CGC GGC          565
Asp Arg Ile Tyr Asn Trp Ala Arg Gln Asn Gly Lys Gln Val Arg Gly
    455                 460                 465

CAC ACC CTG GCC TGG CAC TCG CAG CAG CCG CAG TGG ATG CAG AAC CTC          613
His Thr Leu Ala Trp His Ser Gln Gln Pro Gln Trp Met Gln Asn Leu
470                 475                 480                 485

AGC GGC CAG GCG CTG CGC CAG GCG ATG ATC AAC CAC ATC CAG GGG GTC          661
Ser Gly Gln Ala Leu Arg Gln Ala Met Ile Asn His Ile Gln Gly Val
                490                 495                 500

ATG TCC TAC TAC CGG GGC AAG ATC CCG ATC TGG GAC GTG GTG AAC GAG          709
Met Ser Tyr Tyr Arg Gly Lys Ile Pro Ile Trp Asp Val Val Asn Glu
            505                 510                 515

GCG TTC GAG GAC GGA AAC TCC GGC CGC CGG TGC GAC TCC AAC CTC CAG          757
Ala Phe Glu Asp Gly Asn Ser Gly Arg Arg Cys Asp Ser Asn Leu Gln
        520                 525                 530

CGC ACC GGT AAC GAT TGG ATC GAG GTC GCG TTC CGC ACC GCC CGC CAG          805
Arg Thr Gly Asn Asp Trp Ile Glu Val Ala Phe Arg Thr Ala Arg Gln
    535                 540                 545

GGG GAC CCC TCG GCC AAG CTC TGC TAC AAC GAC TAC AAC ATC GAG AAC          853
Gly Asp Pro Ser Ala Lys Leu Cys Tyr Asn Asp Tyr Asn Ile Glu Asn
550                 555                 560                 565

TGG AAC GCG GCC AAG ACC CAG GCG GTC TAC AAC ATG GTG CGG GAC TTC          901
Trp Asn Ala Ala Lys Thr Gln Ala Val Tyr Asn Met Val Arg Asp Phe
                570                 575                 580

AAG TCC CGC GGC GTG CCC ATC GAC TGC GTG GGC TTC CAG TCG CAC TTC          949
Lys Ser Arg Gly Val Pro Ile Asp Cys Val Gly Phe Gln Ser His Phe
            585                 590                 595

AAC AGC GGT AAC CCG TAC AAC CCG AAC TTC CGC ACC ACC CTG CAG CAG          997
Asn Ser Gly Asn Pro Tyr Asn Pro Asn Phe Arg Thr Thr Leu Gln Gln
        600                 605                 610

TTC GCG GCC CTC GGC GTG GAC GTC GAG GTC ACC GAG CTG GAC ATC GAG         1045
Phe Ala Ala Leu Gly Val Asp Val Glu Val Thr Glu Leu Asp Ile Glu
    615                 620                 625
```

```
AAC GCC CCG GCC CAG ACC TAC GCC AGC GTG ATC CGG GAC TGC CTG GCC          1093
Asn Ala Pro Ala Gln Thr Tyr Ala Ser Val Ile Arg Asp Cys Leu Ala
630                 635                 640                 645

GTG GAC CGC TGC ACC GGC ATC ACC GTC TGG GGT GTC CGC GAC AGC GAC          1141
Val Asp Arg Cys Thr Gly Ile Thr Val Trp Gly Val Arg Asp Ser Asp
                650                 655                 660

TCC TGG CGC TCG TAC CAG AAC CCG CTG CTG TTC GAC AAC AAC GGC AAC          1189
Ser Trp Arg Ser Tyr Gln Asn Pro Leu Leu Phe Asp Asn Asn Gly Asn
            665                 670                 675

AAG AAG CAG GCC TAC TAC GCG GTG CTC GAC GCC CTG AAC GAG GGC TCC          1237
Lys Lys Gln Ala Tyr Tyr Ala Val Leu Asp Ala Leu Asn Glu Gly Ser
        680                 685                 690

GAC GAC GGT GGC GGC CCG TCC AAC CCG CCG GTC TCG CCG CCG CCG GGT          1285
Asp Asp Gly Gly Gly Pro Ser Asn Pro Pro Val Ser Pro Pro Pro Gly
    695                 700                 705

GGC GGT TCC GGG CAG ATC CGG GGC GTG GCC TCC AAC CGG TGC ATC GAC          1333
Gly Gly Ser Gly Gln Ile Arg Gly Val Ala Ser Asn Arg Cys Ile Asp
710                 715                 720                 725

GTG CCG AAC GGC AAC ACC GCC GAC GGC ACC CAG GTC CAG CTG TAC GAC          1381
Val Pro Asn Gly Asn Thr Ala Asp Gly Thr Gln Val Gln Leu Tyr Asp
                730                 735                 740

TGC CAC AGC GGT TCC AAC CAG CAG TGG ACC TAC ACC TCG TCC GGT GAG          1429
Cys His Ser Gly Ser Asn Gln Gln Trp Thr Tyr Thr Ser Ser Gly Glu
            745                 750                 755

TTC CGC ATC TTC GGC AAC AAG TGC CTG GAC GCG GGC GGC TCC AGC AAC          1477
Phe Arg Ile Phe Gly Asn Lys Cys Leu Asp Ala Gly Gly Ser Ser Asn
        760                 765                 770

GGT GCG GTG GTC CAG ATC TAC AGC TGC TGG GGC GGC GCC AAC CAG AAG          1525
Gly Ala Val Val Gln Ile Tyr Ser Cys Trp Gly Gly Ala Asn Gln Lys
    775                 780                 785

TGG GAG CTC CGG GCC GAC GGC ACC ATC GTG GGC GTG CAG TCC GGG CTG          1573
Trp Glu Leu Arg Ala Asp Gly Thr Ile Val Gly Val Gln Ser Gly Leu
790                 795                 800                 805

TGC CTC GAC GCG GTG GGT GGC GGC ACC GGC AAC GGC ACG CGG CTG CAG          1621
Cys Leu Asp Ala Val Gly Gly Gly Thr Gly Asn Gly Thr Arg Leu Gln
                810                 815                 820

CTC TAC TCC TGC TGG GGC GGC AAC AAC CAG AAG TGG TCC TAC AAC GCC          1669
Leu Tyr Ser Cys Trp Gly Gly Asn Asn Gln Lys Trp Ser Tyr Asn Ala
            825                 830                 835
```

TGA TCCCCGGCTG ATCGACCCTA GTTGAGGCCG TCTCCGGTAC GGCACCGTCG 1722

GACCGGAGGC GGTCCCTTGT TCGTCCAGGA CGGAAGGACC GGTCTGAGCA GGCGCGGCGA 1782

TCGGACACCA TGGTGGGAGG CACGAAAGCG GGAGGGGGTC GTATTCCGAG ACTCCGGGAA 1842

GTGGAGGTGT TCCTCCACCT GA 1864

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 493 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Gly Val Asn Ala Phe Pro Arg Pro Gly Ala Arg Arg Phe Thr Gly
 1               5                  10                  15

Gly Leu Tyr Arg Ala Leu Ala Ala Ala Thr Val Ser Val Val Gly Val
            20                  25                  30

Val Thr Ala Leu Thr Val Thr Gln Pro Ala Ser Ala Ala Ala Ser Thr
        35                  40                  45

Leu Ala Glu Gly Ala Ala Gln His Asn Arg Tyr Phe Gly Val Ala Ile
    50                  55                  60

Ala Ala Asn Arg Leu Thr Asp Ser Val Tyr Thr Asn Ile Ala Asn Arg
65                  70                  75                  80

Glu Phe Asn Ser Val Thr Ala Glu Asn Glu Met Lys Ile Asp Ala Thr
                85                  90                  95

Glu Pro Gln Gln Gly Arg Phe Asp Phe Thr Gln Ala Asp Arg Ile Tyr
            100                 105                 110

Asn Trp Ala Arg Gln Asn Gly Lys Gln Val Arg Gly His Thr Leu Ala
        115                 120                 125

Trp His Ser Gln Gln Pro Gln Trp Met Gln Asn Leu Ser Gly Gln Ala
    130                 135                 140

Leu Arg Gln Ala Met Ile Asn His Ile Gln Gly Val Met Ser Tyr Tyr
145                 150                 155                 160

Arg Gly Lys Ile Pro Ile Trp Asp Val Val Asn Glu Ala Phe Glu Asp
                165                 170                 175
```

Gly Asn Ser Gly Arg Arg Cys Asp Ser Asn Leu Gln Arg Thr Gly Asn
180 185 190

Asp Trp Ile Glu Val Ala Phe Arg Thr Ala Arg Gln Gly Asp Pro Ser
195 200 205

Ala Lys Leu Cys Tyr Asn Asp Tyr Asn Ile Glu Asn Trp Asn Ala Ala
210 215 220

Lys Thr Gln Ala Val Tyr Asn Met Val Arg Asp Phe Lys Ser Arg Gly
225 230 235 240

Val Pro Ile Asp Cys Val Gly Phe Gln Ser His Phe Asn Ser Gly Asn
245 250 255

Pro Tyr Asn Pro Asn Phe Arg Thr Thr Leu Gln Gln Phe Ala Ala Leu
260 265 270

Gly Val Asp Val Glu Val Thr Glu Leu Asp Ile Glu Asn Ala Pro Ala
275 280 285

Gln Thr Tyr Ala Ser Val Ile Arg Asp Cys Leu Ala Val Asp Arg Cys
290 295 300

Thr Gly Ile Thr Val Trp Gly Val Arg Asp Ser Asp Ser Trp Arg Ser
305 310 315 320

Tyr Gln Asn Pro Leu Leu Phe Asp Asn Asn Gly Asn Lys Lys Gln Ala
325 330 335

Tyr Tyr Ala Val Leu Asp Ala Leu Asn Glu Gly Ser Asp Asp Gly Gly
340 345 350

Gly Pro Ser Asn Pro Pro Val Ser Pro Pro Pro Gly Gly Gly Ser Gly
355 360 365

Gln Ile Arg Gly Val Ala Ser Asn Arg Cys Ile Asp Val Pro Asn Gly
370 375 380

Asn Thr Ala Asp Gly Thr Gln Val Gln Leu Tyr Asp Cys His Ser Gly
385 390 395 400

Ser Asn Gln Gln Trp Thr Tyr Thr Ser Ser Gly Glu Phe Arg Ile Phe
405 410 415

Gly Asn Lys Cys Leu Asp Ala Gly Gly Ser Ser Asn Gly Ala Val Val
420 425 430

Gln Ile Tyr Ser Cys Trp Gly Gly Ala Asn Gln Lys Trp Glu Leu Arg
435 440 445

Ala Asp Gly Thr Ile Val Gly Val Gln Ser Gly Leu Cys Leu Asp Ala
450 455 460

```
Val Gly Gly Gly Thr Gly Asn Gly Thr Arg Leu Gln Leu Tyr Ser Cys
465                 470                 475                 480

Trp Gly Gly Asn Asn Gln Lys Trp Ser Tyr Asn Ala  *
                485                 490
```

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..6
(D) OTHER INFORMATION:/label= AM35_N-term

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Asp Thr Thr Ile Thr Gln
1               5
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..14
(D) OTHER INFORMATION:/label= AM50_1696_pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Ala Ala Ser Thr Leu Ala Glu Gly Ala Ala Gln His Asn Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..10
(D) OTHER INFORMATION:/label= AM50_1697_pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

Tyr Phe Gly Val Ala Ile Ala Ala Asn Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..12
(D) OTHER INFORMATION:/label= AM50_1698_pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

Leu Asn Asp Ser Val Tyr Thr Asn Ile Ala Asn Arg
1 5 10

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..9
(D) OTHER INFORMATION:/label= AM50_1699_pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

Xaa Thr Gly Ile Thr Val Xaa Gly Val
1 5

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Actinomadura flexuosa
(B) STRAIN: DSM43186

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..11
(D) OTHER INFORMATION:/label= AM50_1704_pep

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

Glu Phe Asn Ser Val Thr Ala Glu Asn Glu Met
1 5 10

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichoderma reesei and Actinomadura flexuosa
(B) STRAIN: QM6a and DSM43186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..18
(D) OTHER INFORMATION:/product= "Mannanase-xylanase fusion protein"
/note= "Partial sequence of the fusion in pALK945. Bases 1-5 are bases 1342-1346 of T.reesei man1 sequence, bases 6-9 synthetic and bases 10-18 are bases 432-440 of A.flexuosa AM35 sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

TAT GGT CGC GAC ACC ACC
Tyr Gly Arg Asp Thr Thr 18
495

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

Tyr Gly Arg Asp Thr Thr
1 5

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichoderma reesei and Actinomadura flexuosa
(B) STRAIN: QM6a and DSM43186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..27
(D) OTHER INFORMATION:/product= "Mannanase-xylanase fusion protein"
/note= "Partial sequence of the fusion in pALK948. Bases 1-5 are bases 1342-1346 of T.reesei man1 sequence, bases 6-18 are synthetic KEX2-linker, bases are 19-27 are bases 432-440 cf A.flexuosa AM35 sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
TAT GGT CGC GAC AAG CGC GAC ACC ACC                                    27
Tyr Gly Arg Asp Lys Arg Asp Thr Thr
 10                  15
```

(2) INFORMATION FOR SEQ ID NO: 14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

```
Tyr Gly Arg Asp Lys Arg Asp Thr Thr
 1               5
```

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichoderma reesei and Actinomadura flexuosa
(B) STRAIN: QM6a and DSM43186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..39
(D) OTHER INFORMATION:/product= "Mannanase-xylanase fusion protein"
/note= "Partial sequence of the fusion in pALK1021. Bases 1-18 are bases 1342-1359 of T.reesei man1 sequence, and bases 19-39 are bases 432-452 of A.flexuosa AM35 sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
TAT GGC CAG TGT GGA GGT GAC ACC ACC ATC ACC CAG AAC                    39
Tyr Gly Gln Cys Gly Gly Asp Thr Thr Ile Thr Gln Asn
 10                  15                  20
```

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
Tyr Gly Gln Cys Gly Gly Asp Thr Thr Ile Thr Gln Asn
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Trichoderma reesei and Actinomadura flexuosa
(B) STRAIN: QM6a and DSM43186

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..39
(D) OTHER INFORMATION:/product= "Mannanase-xylanase fusion protein"
/note= "Partial sequence of the fusion in pALK1022. Bases 1-18 are bases 1342-1359 of T.reesei man1 sequence, bases 19-30 are synthetic KEX2-linker, bases 31-39 are bases 432-440 of A.flexuosa AM35 sequence."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
TAT GGC CAG TGT GGA GGT CGC GAC AAG CGC GAC ACC ACC                    39
Tyr Gly Gln Cys Gly Gly Arg Asp Lys Arg Asp Thr Thr
     15                  20                  25
```

(2) INFORMATION FOR SEQ ID NO: 18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
Tyr Gly Gln Cys Gly Gly Arg Asp Lys Arg Asp Thr Thr
 1               5                  10
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 10, line last paragraph.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Deutsche Sammlung von Mikroorganismen und Zellkulturen

Address of depositary institution (*including postal code and country*)

Mascheroder Weg 1 b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
03.04.1995	DSM9899

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

Regarding those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4 EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn. This request also applies to other designated countries in which similar or corresponding provisions are in force.

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B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
Deutsche Sammlung von Mikroorganismen und Zellkulturen

Address of depositary institution (*including postal code and country*)
Mascheroder Weg 1 b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
03.04.1995	DSM9900

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E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

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Date of deposit	Accession Number
03.04.1995	DSM9902

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Date of deposit	Accession Number
03.04.1995	DSM9903

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Deutsche Sammlung von Mikroorganismen und Zellkulturen

Address of depositary institution *(including postal code and country)*
Mascheroder Weg 1 b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
27.09.1994	DSM9447

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

Regarding those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(4) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn. This request also applies to other designated countries in which similar or corresponding provisions are in force.

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Address of depositary institution (*including postal code and country*)
Mascheroder Weg 1 b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
27.09.1994	DSM9448

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

Regarding those designations in which a European patent is sought, a sample of the deposited microorganism will be made available only by the issue of such a sample to an expert nominated by the person requesting the sample (Rule 28(2) EPC) until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or is deemed to be withdrawn. This request also applies to other designated countries in which similar or corresponding provisions are in force.

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B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

Deutsche Sammlung von Mikroorganismen und Zellkulturen

Address of depositary institution *(including postal code and country)*

Mascheroder Weg 1 b, D-38124 Braunschweig, Germany

Date of deposit	Accession Number
27.07.1997	DSM9322

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

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What Is Claimed Is:

1. A recombinant expression vector for production of bacterial proteins in a filamentous fungal host comprising a promoter operably linked to a DNA sequence of a filamentous fungus secretable protein or one or more functional domains of said protein, which is fused in frame with a DNA sequence encoding a bacterial protein.

2. The recombinant expression vector of claim 1, wherein the filamentous fungus secretable protein encoding DNA sequence is a DNA sequence encoding an enzyme, most preferably cellulase or hemicellulase homologous or heterologous to the secreting filamentous fungus, preferably *Trichoderma* or *Aspergillus*, most preferably *T.reesei*.

3. The recombinant expression vector of claim 2, wherein the DNA sequence encoding the filamentous fungus secretable protein is selected from the group consisting of *Aspergillus* glucoamylase, *Aspergillus* α-amylase, *Trichoderma* cellulase, *Trichoderma* hemicellulase, *Trichoderma* glucoamylase, *Hormoconis* glucoamylase, *Chaetomium* xylanase, and *Melanocarpus* cellulase.

4. The recombinant expression vector of claim 3, wherein the DNA sequence encoding the filamentous fungus secretable protein of *Trichoderma* is a homologous enzyme selected from the group consisting of cellobiohydrolases (CBHI and CBHII), endoglucanases (EGI and EGII), xylanases (XYLI and XYLII) and mannanase (MANI).

5. The recombinant expression vector of claim 1, wherein the bacterial protein encoding DNA sequence is originating from actinomycete.

6. The recombinant expression vector of claim 1, wherein the said DNA sequence encoding the bacterial protein is a DNA sequence encoding an enzyme, preferably a xylanase or cellulase originating from bacteria, most preferably a xylanase or cellulase originating from an actinomycete.

7. The recombinant expression vector of claim 1, wherein said bacterial protein encoding DNA sequence comprises the DNA sequence SEQ ID NO: 1: encoding the amino acid sequence of SEQ ID NO: 2: or an equivalent of said amino acid sequence, wherein said equivalent has a xylanolytic activity.

8. The recombinant expression vector of claim 1, wherein said bacterial protein encoding DNA sequence comprises the DNA sequence SEQ ID NO: 3: encoding the amino acid sequence of SEQ ID NO: 4: or an equivalent of said amino acid sequence, wherein said equivalent has a xylanolytic activity.

9. The recombinant expression vector of claim 1, wherein the bacterial protein encoding DNA sequence is comprised in plasmids selected from the group consisting of pALK923 (DSM9322), pALK938 (DSM9899), pALK939 (DSM9900), pALK940 (DSM9901), pALK941 (DSM9902) and pALK1056 (DSM9903) that encodes *Actinomadura flexuosa* AM35 xylanase .

10. The recombinant expression vector of claim 1, wherein the bacterial protein encoding DNA sequence is comprised in plasmids selected from a group consisting of plasmid pALK927 (DSM9447) or plasmid pALK928 (DSM9448) that encodes the *Actinomadura flexuosa* AM50 xylanase.

11. The recombinant expression vector of claim 1, wherein an isolated DNA sequence encodes *Thermomonospora fusca* cellulases, most preferably *T.fusca* endocellulase E5.

12. The recombinant expression vector of claim 1 comprising an isolated DNA sequence encoding for a bacterial protein, preferably an enzyme, most preferably a xylanase or a cellulase or equivalents thereof, wherein said isolated DNA sequence is operably linked to a promoter of a filamentous fungus secretable protein, most preferably to a *T. reesei cbh1* promoter or to a *A. niger* glucoamylase promoter.

13. The recombinant expression vector of claim 12, wherein the isolated DNA sequence encoding the xylanase is SEQ ID NO: 1: or SEQ ID NO: 3: or equivalents thereof.

14. The recombinant expression vector of claim 1, wherein said expression vector comprises the plasmid construct of Figure 17, wherein plasmid pALK945 carries the SEQ ID NO: 11:, plasmid pALK948 carries SEQ ID NO: 13:, plasmid pALK1021 carries SEQ ID NO: 15: and plasmid pALK1022 (Figure 17) carries SEQ ID NO: 17: or equivalents thereof encoding the amino acid sequences SEQ ID NO: 12:, SEQ ID NO: 14:, SEQ ID NO: 16:. SEQ ID NO: 18:, or equivalents thereof.

15. A filamentous fungus host, transformed with at least one of the expression vectors of claims 1-14.

16. The filamentous fungus host of claim 15, wherein the filamentous fungus is selected from a group consisting of *Trichoderma* and *Aspergillus*, more preferably of *Trichoderma*, most preferably *T. reesei*.

17. A preparation comprising one or more bacterial proteins or enzymes, preferably xylanases or cellulases secreted into the culture medium during the cultivation of a filamentous fungus, preferably a host of claims 15-16.

18. The preparation of claim 17, wherein the preparation comprises spent culture medium essentially free from host cells.

19. The preparation of claims 17-18, wherein the preparation comprises a protein or enzyme, preferably a xylanase or cellulase obtainable from the spent culture medium by filtration, ultrafiltration, centrifugation, drying, evaporation, precipitation, immobilization or any other downstream processing method.

20. The preparation of claim 19, wherein the protein or enzyme is further isolated and purified.

21. A method for enzyme-aided bleaching in processing temperatures, said method comprising adding a bacterial xylanase containing preparation of claims 17-20 to pulp.

22. The method of claim 21, wherein the processing temperature is 50-90 °C, preferably 60-85 °C, most preferably 70-80 °C.

23. A method for treating of paper making pulp, said method comprising adding a bacterial xylanase containing preparation of claims 17-20 to pulp.

24. A method for enzymatically treating plant biomass, which comprises contacting said biomass with a bacterial xylanase containing preparation of claims 17-20.

25. A method for producing a preparation containing an enzyme originating from bacteria preferably from actinomycete in a filamentous fungal host comprising the steps of:

(a) constructing a recombinant expression vector, said vector having a promoter operably linked to a DNA sequence of a filamentous fungus secretable protein or one or more functional domains of said protein, which is fused in frame with a DNA sequence encoding

a bacterial protein:

(b) transforming a filamentous fungal host with the vector from step (a):

(c) cultivating the host of step (b) in a culture medium and under culture condition suitable for the secretion of the enzyme;

(d) separating the host cells from the culture medium of step (c) when a desired production level is achieved; and

(e) subjecting the essentially cell-free medium of step (d) to down-stream processing methods compatible to the use of the preparation and giving sufficient storability and stability to the preparation.

26. An isolated DNA sequence encoding the amino acid sequence of *Actinomadura* xylanase, comprising the amino acid sequence of SEQ ID NO: 2: or SEQ ID NO: 4: or an equivalent of said amino acid sequence, wherein said equivalent has the biological activity of said xylanase comprising the sequence of SEQ ID NO: 2: or SEQ ID NO: 4:.

27. An isolated DNA fragment of a plasmid comprising the DNA sequences selected from the group consisting of pALK923 (DSM9322), pALK938 (DSM9899), pALK939 (DSM9900), pALK940 (DSM9901), pALK941 (DSM9902) and pALK1056 (DSM9903) that encodes *Actinomadura flexuosa* AM35 xylanase and equivalents thereof.

28. An isolated DNA fragment of plasmid comprising the DNA sequences selected from the plasmids selected from a group consisting of plasmid pALK927 (DSM9447) or plasmid pALK928 (DSM9448) that encodes the *Actinomadura flexuosa* AM50 xylanase and equivalents thereof.

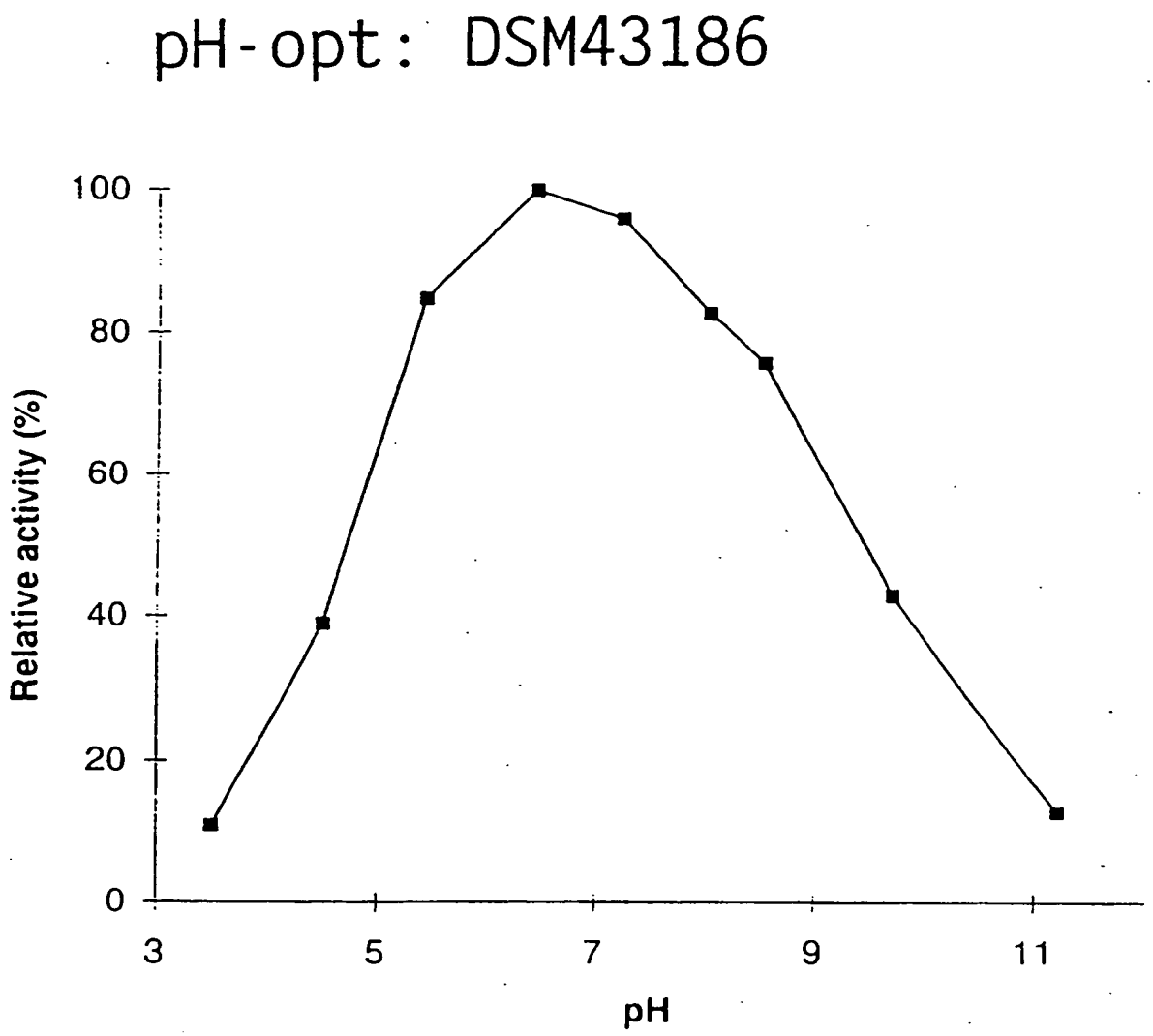

FIGURE 1

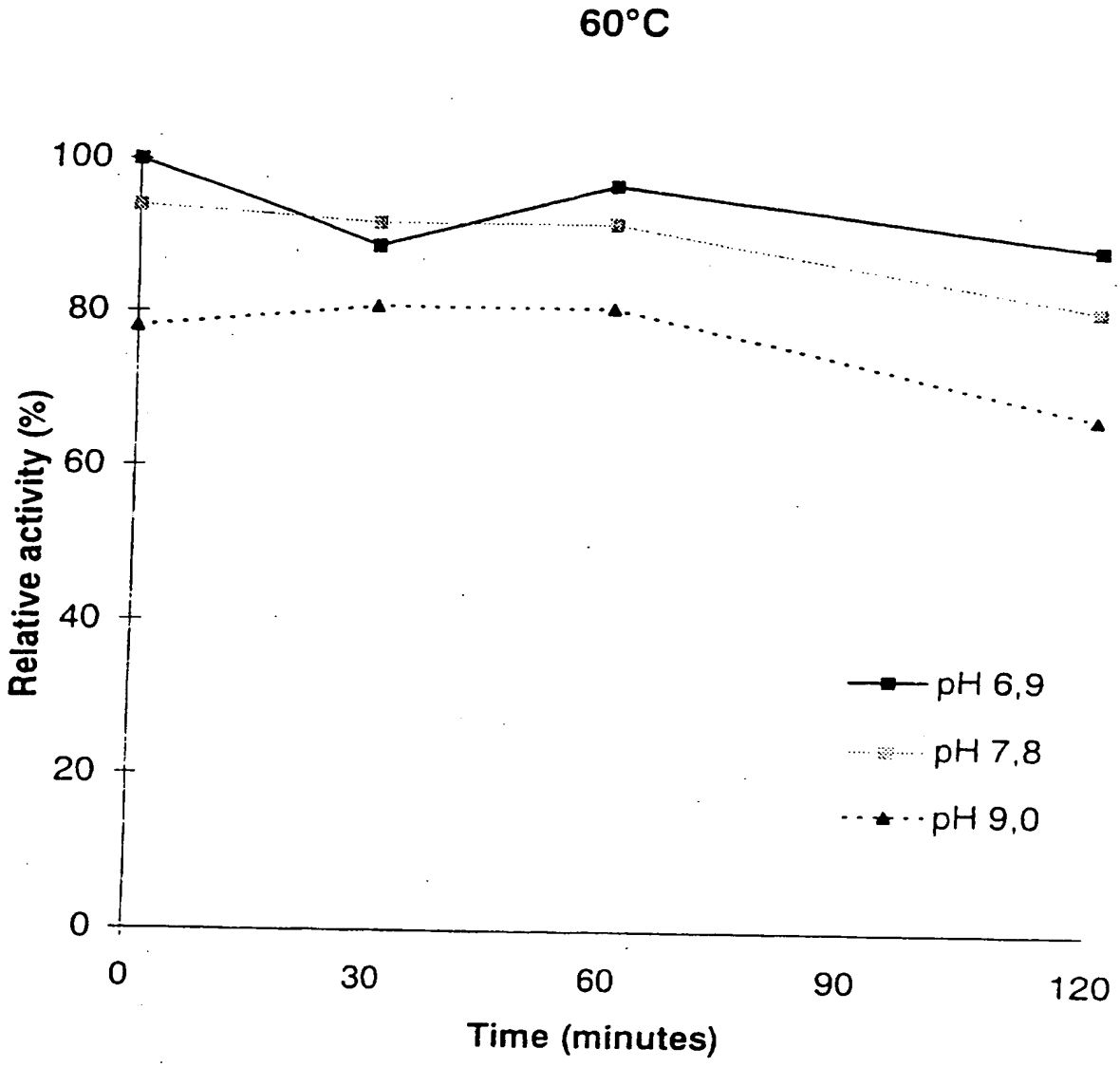
60°C
100
80
60
40
20
0
Relative activity (%)
0
30
60
90
120
Time (minutes)
pH 6,9
pH 7,8
pH 9,0

FIGURE 2A

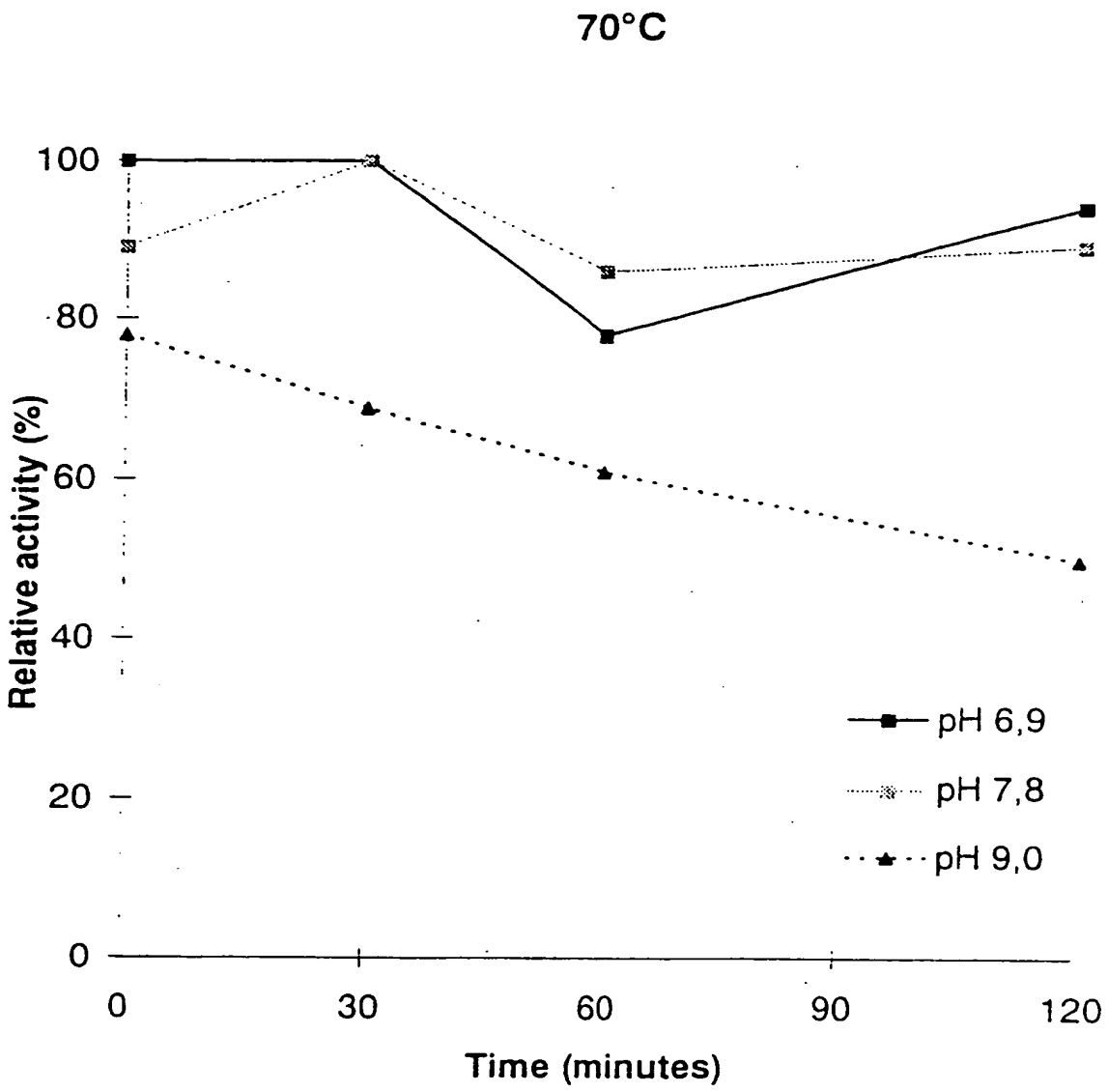
70°C
100
80
60
40
20
0
Relative activity (%)
0
30
60
90
120
Time (minutes)
pH 6,9
pH 7,8
pH 9,0

FIGURE 2B

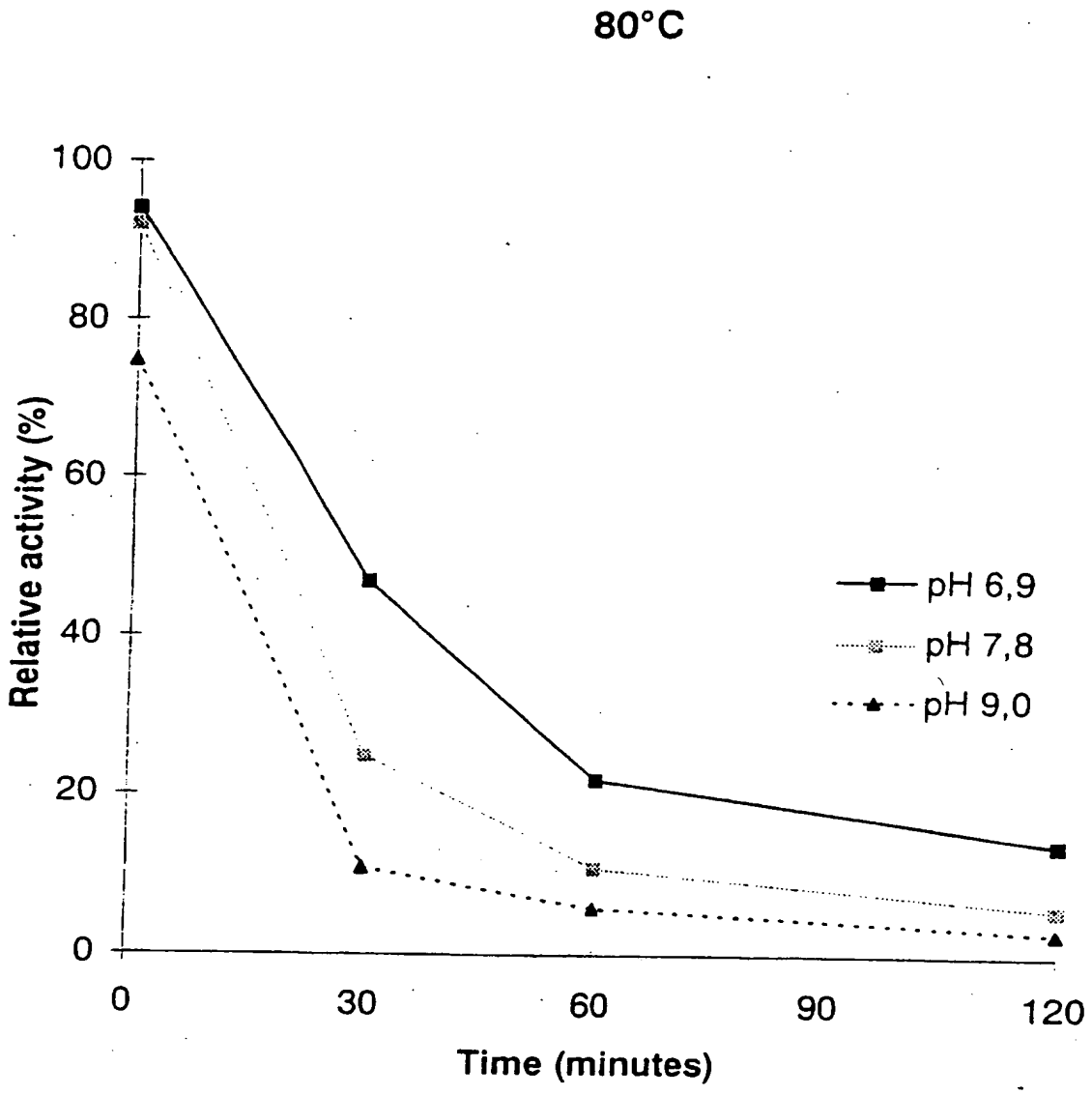
80°C
100
80
60
40
20
0
Relative activity (%)
0
30
60
90
120
Time (minutes)
pH 6,9
pH 7,8
pH 9,0

FIGURE 2C

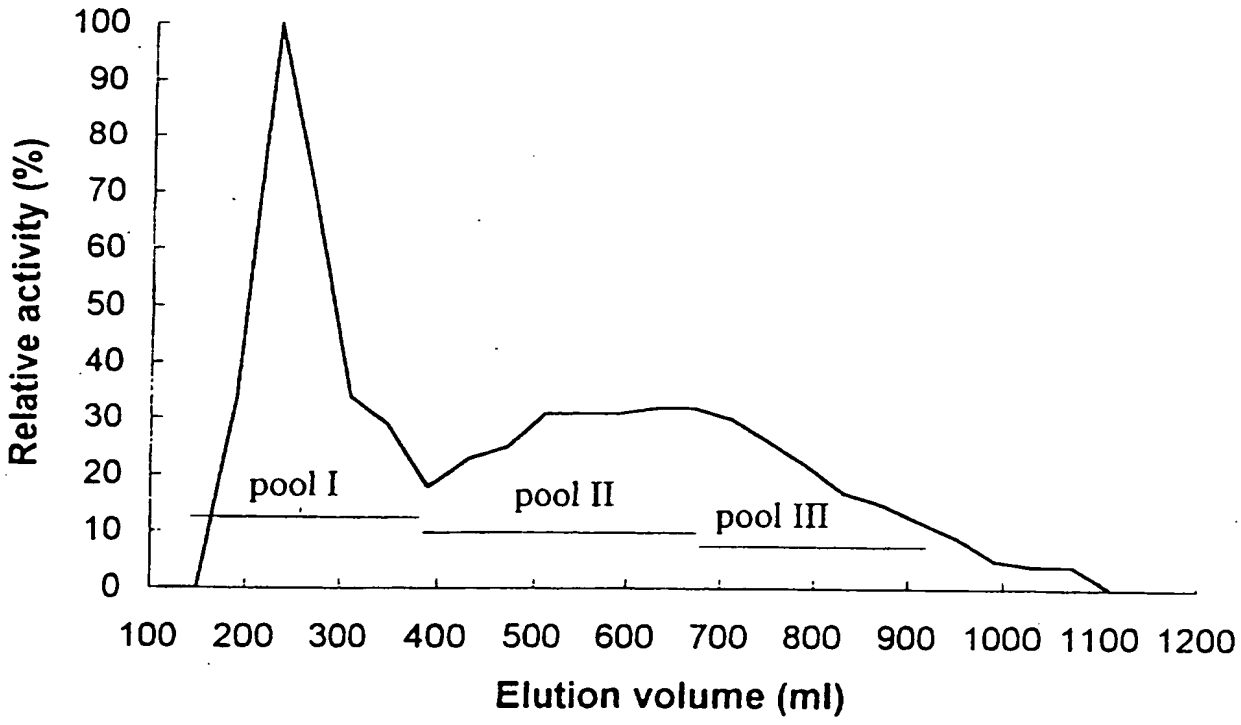
Relative activity (%)
100
90
80
70
60
50
40
30
20
10
0
pool I
pool II
pool III
100 200 300 400 500 600 700 800 900 1000 1100 1200
Elution volume (ml)

FIGURE 3

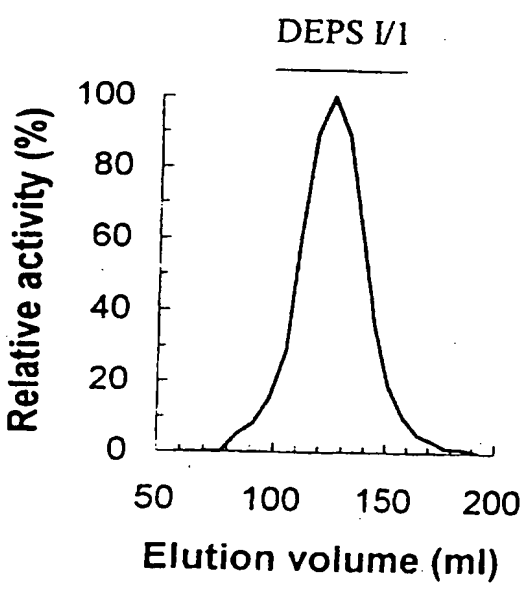

FIGURE 4A

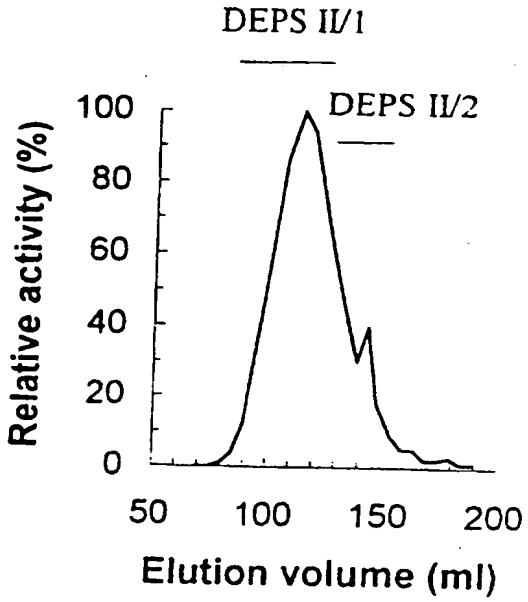

FIGURE 4B

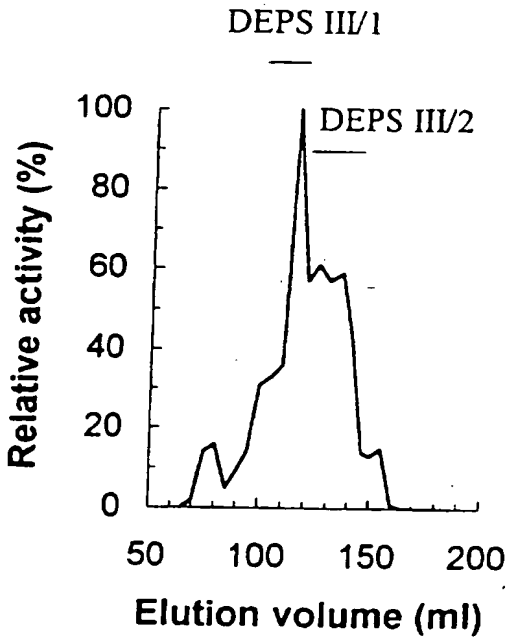

FIGURE 4C

LANE

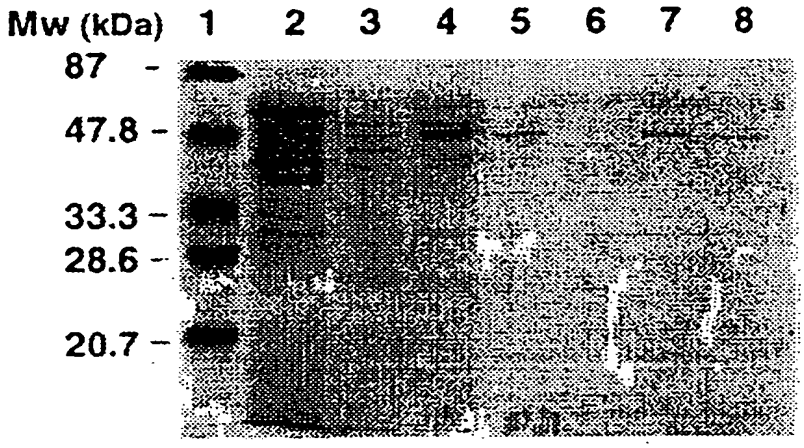

FIGURE 5A

LANE

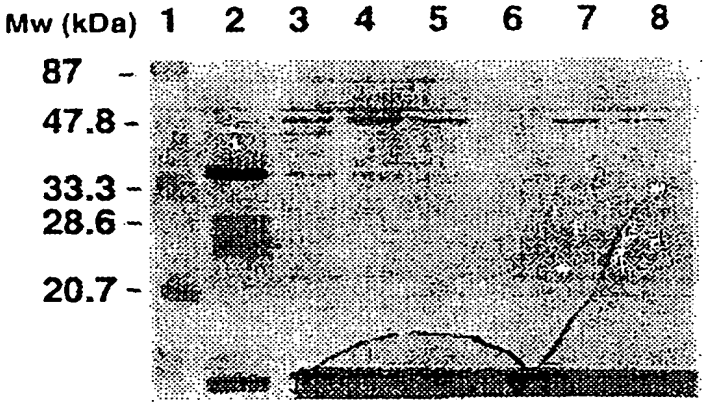

FIGURE 5B

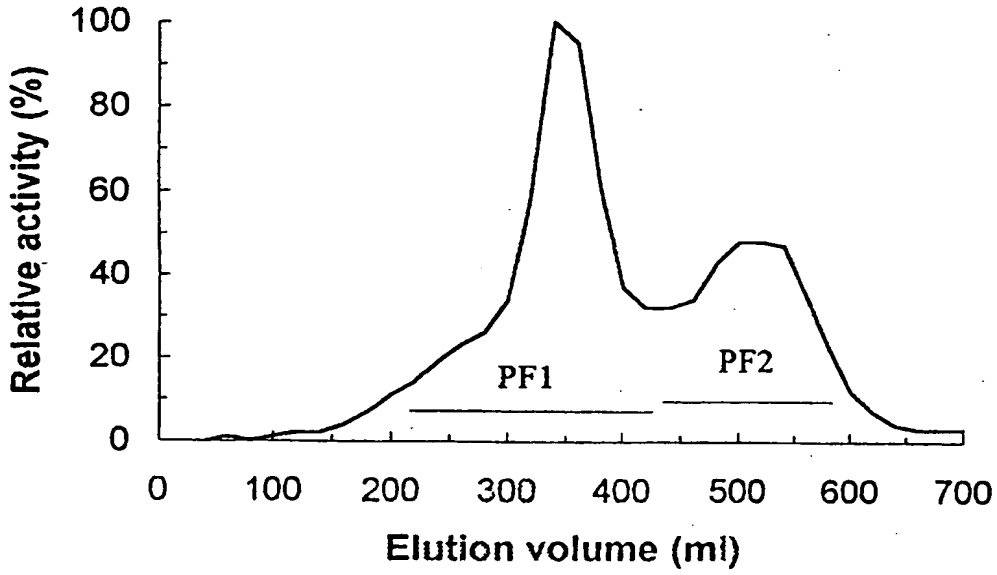

FIGURE 6A

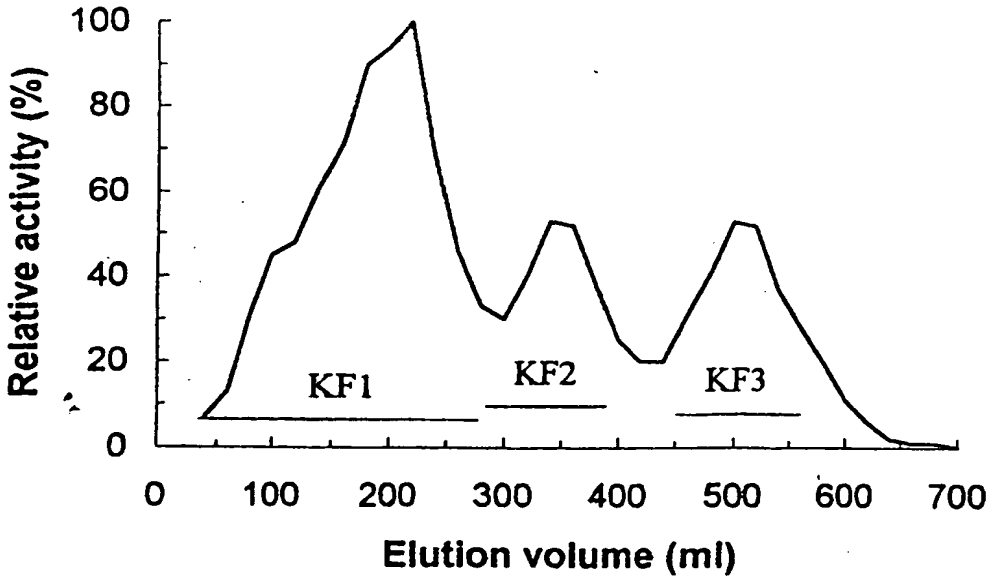

FIGURE 6B

LANE

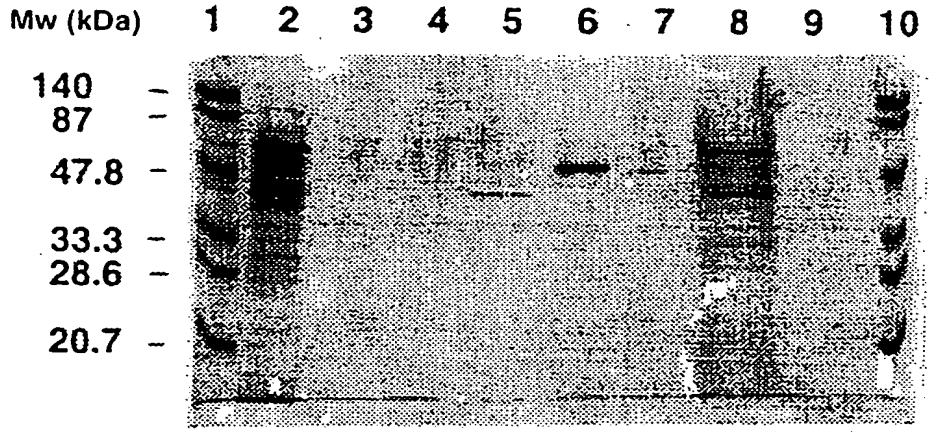

FIGURE 7A

LANE

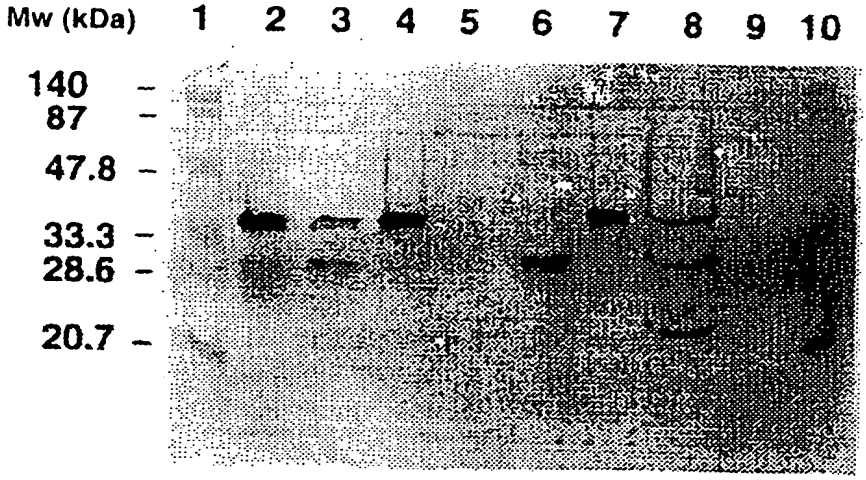

FIGURE 7B

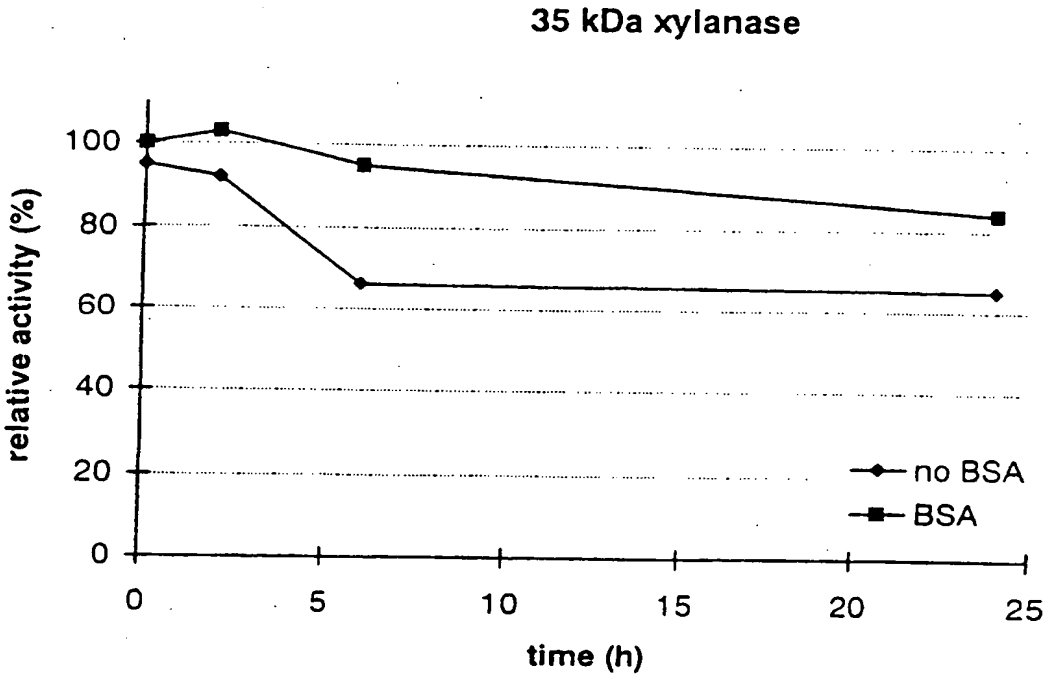
35 kDa xylanase
relative activity (%)
100
80
60
40
20
0
0
5
10
15
20
25
time (h)
no BSA
BSA

FIGURE 8

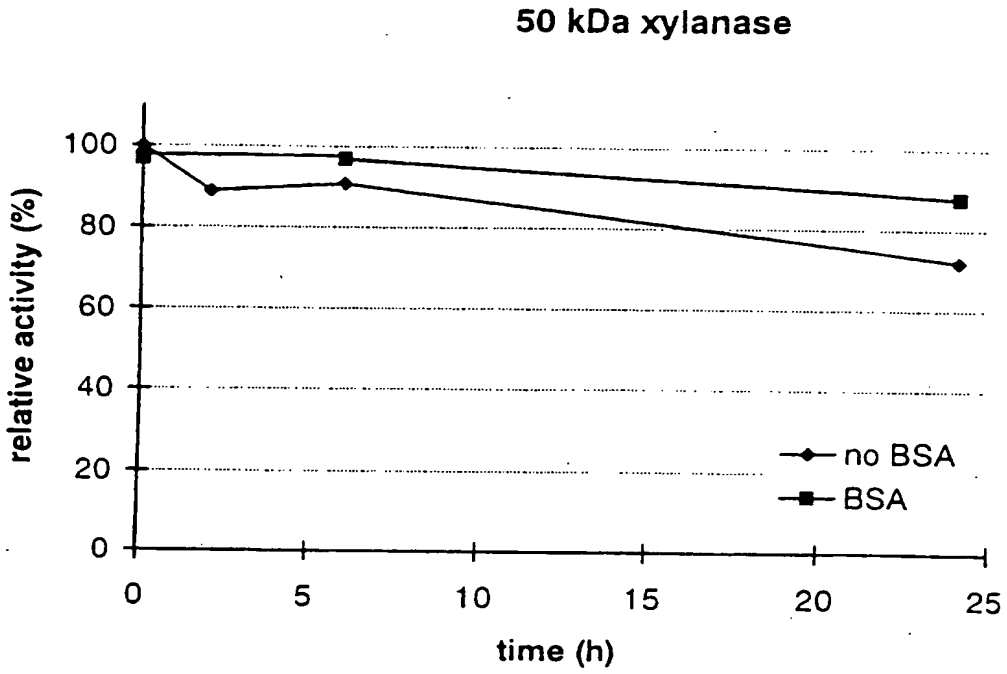
50 kDa xylanase
relative activity (%)
100
80
60
40
20
0
0
5
10
15
20
25
time (h)
no BSA
BSA

FIGURE 9

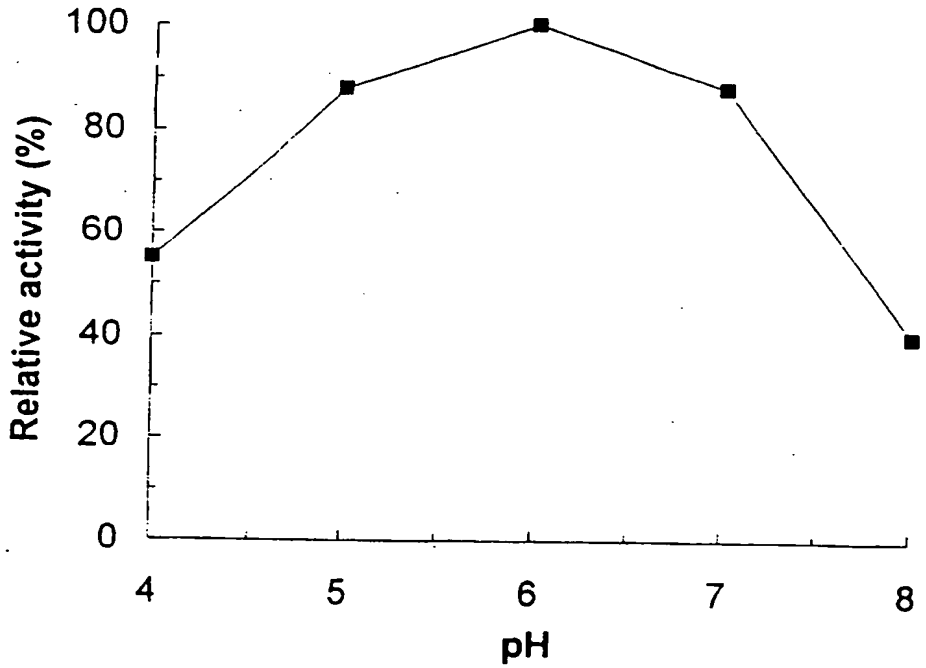

FIGURE 10A

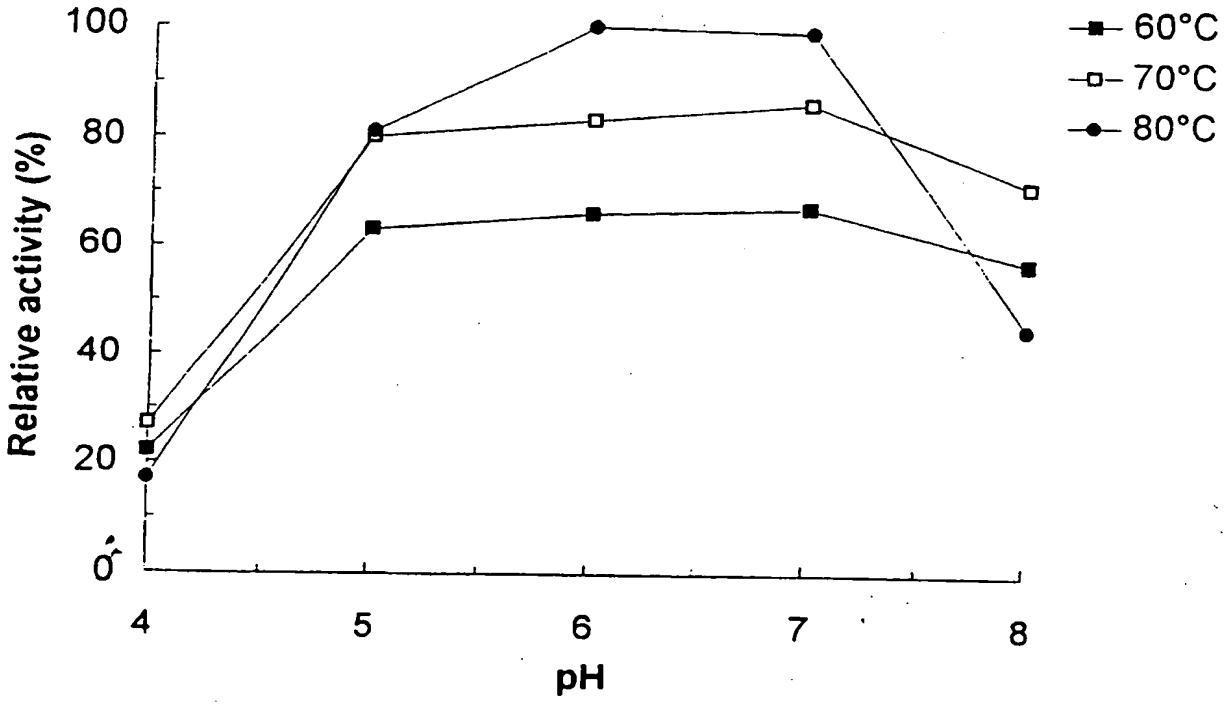

FIGURE 10B

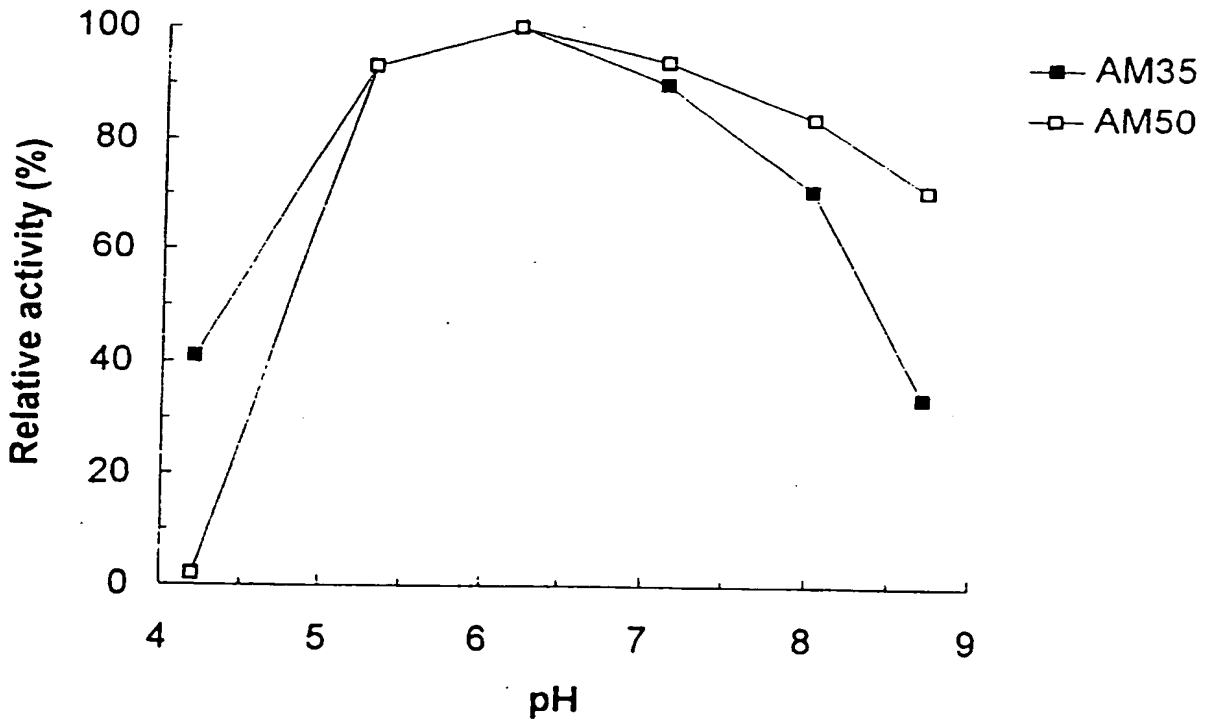

FIGURE 10C

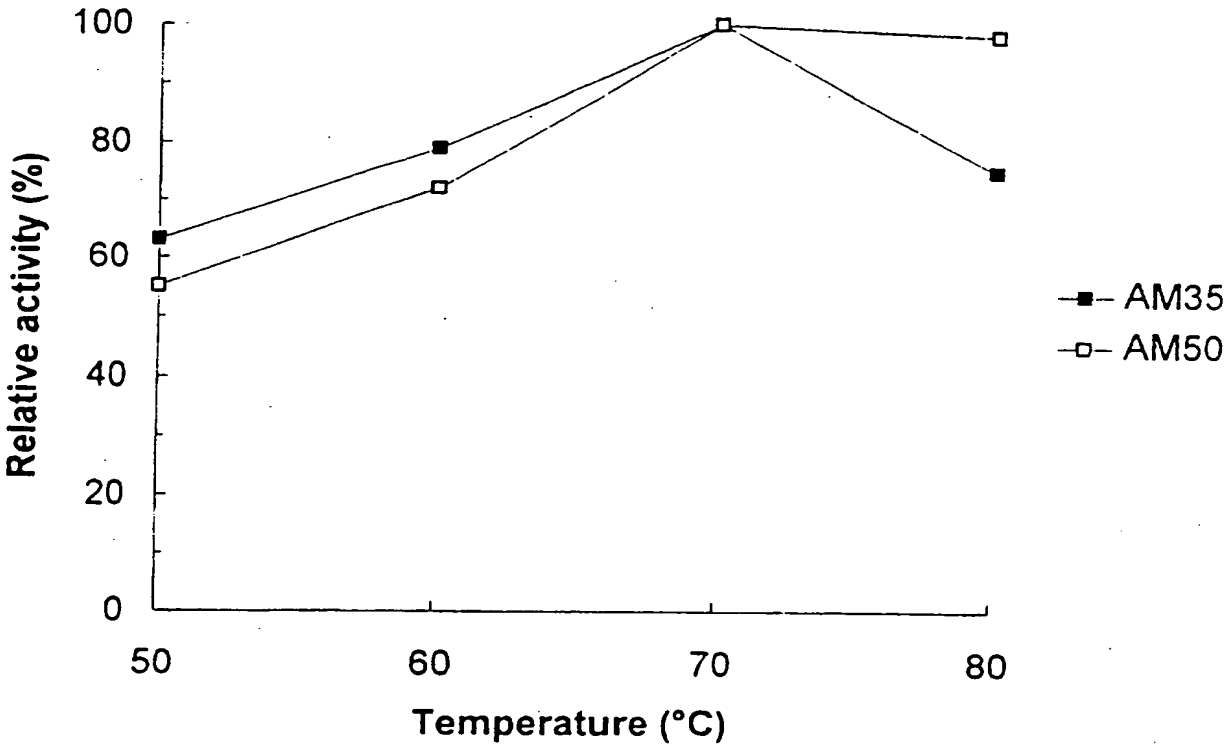
100
80
60
40
20
0
Relative activity (%)
50
60
70
80
Temperature (°C)
AM35
AM50

FIGURE 11

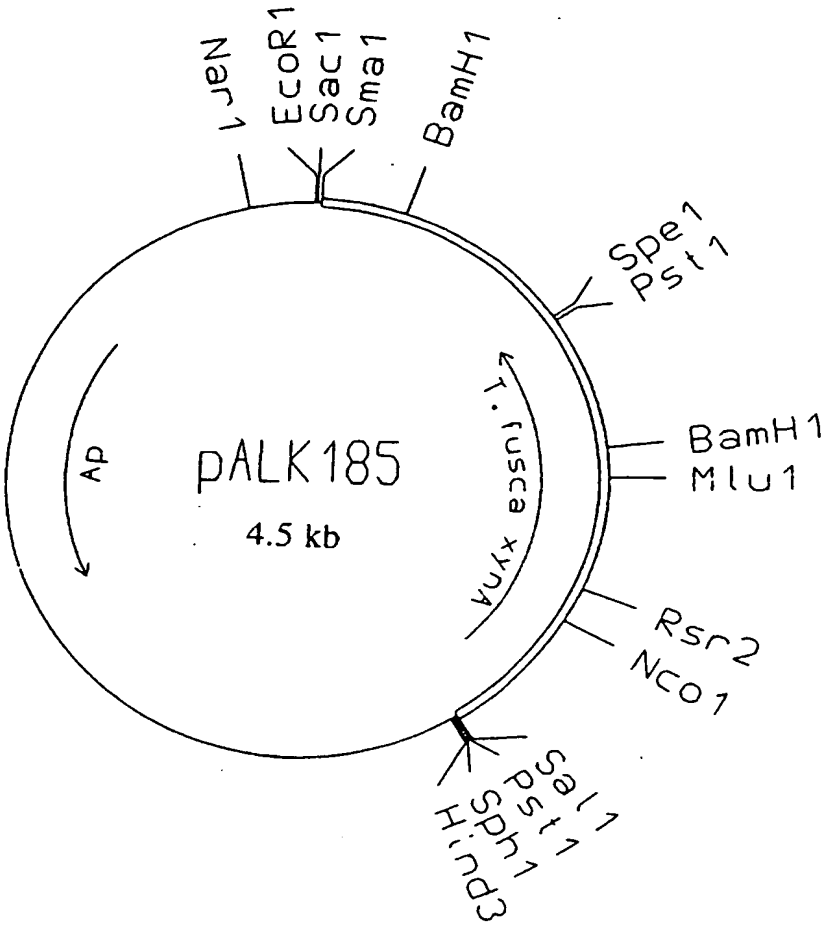
NdeI
EcoR1
Sac1
Sma1
BamH1
Spe1
Pst1
BamH1
Mlu1
Rsr2
NCO1
Sal1
Pst1
Sph1
Hind3
Ap
pALK185
4.5 kb
T. fusca xyna

FIGURE 12

```
1    CCCGGGTATTCATGTGAATGATTAGCAACAGTTATGTTACGGAGATATTTCTGAGAGTGTTGACAGGTCGTGAAGTCGGT   80

81   CCGATACTTTCGAGCTAGCTCCGATAGTTTTCGATACGCCGGCACATCGAGCACGTCGGACGAGTCACGCGCCACGTCGG  160

161  TTTTCCGCCGCACGCCGCGCAGAGCGGCCGGAGAACCCCCGCGTGTCCGCGGCATCGGTGCCGGTCCGTCGTTCGCCGCC  240

241  GACCGCGCGCCCGGGTCGCGACACGCCAGCCCCCATCGGCCCTTCTTCACGAGGAAGCCGTACATGAACGAACCCCTCACC  320
                                                                  M  N  E  P  L  T

321  ATCACGCAGGCCAGGCGCCGCAGACGCCTCGGCCTCCGGCGCATCGTCACCAGTGCCTTCGCCCTGGCACTCGCCATCGC  400
     I  T  Q  A  R  R  R  R  R  L  G  L  R  R  I  V  T  S  A  F  A  L  A  L  A  I  A

401  CGGTGCGCTGCTGCCCGGCACGGCCCACGCCGACACCACCATCACCCAGAACCAGACCGGGTACGACAACGGCTACTTCT  480
     G  A  L  L  P  G  T  A  H  A  D  T  T  I  T  Q  N  Q  T  G  Y  D  N  G  Y  F  Y

481  ACTCGTTCTGGACCGACGCGCCCGGGACCGTCTCCATGACCCTCCACTCGGGCGGCAGCTACAGCACCTCGTGGCGGAAC  560
       S  F  W  T  D  A  P  G  T  V  S  M  T  L  H  S  G  G  S  Y  S  T  S  W  R  N

561  ACCGGGAACTTCGTCGCCGGCAAGGGCTGGTCCACCGGGGGACGGCGGACCGTGACCTACAACGCCTCCTTCAACCCGTC  640
     T  G  N  F  V  A  G  K  G  W  S  T  G  G  R  R  T  V  T  Y  N  A  S  F  N  P  S

641  GGGTAACGGCTACCTCACGCTCTACGGCTGGACCAGGAACCCGCTCGTCGAGTACTACATCGTCGAGAGCTGGGGCACCT  720
       G  N  G  Y  L  T  L  Y  G  W  T  R  N  P  L  V  E  Y  Y  I  V  E  S  W  G  T  Y

721  ACCGGCCCACCGGCACCTACAAGGGCACCGTCACCACCGACGGGGGAACGTACGACATCTACGAGACCTGGCGGTACAAC  800
       R  P  T  G  T  Y  K  G  T  V  T  T  D  G  G  T  Y  D  I  Y  E  T  W  R  Y  N

801  GCGCCGTCCATCGAGGGCACCCGGACCTTCCAGCAGTTCTGGAGCGTCCGGCAGCAGAAGCGGACCAGCGGCACCATCAC  880
     A  P  S  I  E  G  T  R  T  F  Q  Q  F  W  S  V  R  Q  Q  K  R  T  S  G  T  I  T

881  CATCGGCAACCACTTCGACGCCTGGGCCCGCGCCGGCATGAACCTGGGCAGCCACGACTACCAGATCATGGCGACCGAGG  960
       I  G  N  H  F  D  A  W  A  R  A  G  M  N  L  G  S  H  D  Y  Q  I  M  A  T  E  G

961  GCTACCAGAGCAGCGGTAGCTCCACCGTCTCCATCAGCGAGGGTGGCAACCCCGGCAACCCGGGTAACCCCGGCAACCCC  1040
       Y  Q  S  S  G  S  S  T  V  S  I  S  E  G  G  N  P  G  N  P  G  N  P  G  N  P

1041 GGCAACCCCGGTAACCCGGGTAACCCCGGCGGTGGCTGCGTCGCGACCCTCTCCGCCGGCCAGCAGTGGAGCGACCGCTA  1120
     G  N  P  G  N  P  G  N  P  G  G  G  C  V  A  T  L  S  A  G  Q  Q  W  S  D  R  Y

1121 CAACCTCAACGTCTCGGTCAGCGGCTCGAACAACTGGACGGTCCGGATGGACGTGCCCTACCCGGCCCGCATCATCGCCA  1200
       N  L  N  V  S  V  S  G  S  N  N  W  T  V  R  M  D  V  P  Y  P  A  R  I  I  A  T

1201 CCTGGAACATCCACGCCCAGTGGCCCGAGTCCCAGGTGCTCATCGCCAGACCCAACGGCAACGGCAACAACTGGGGCGTG  1280
       W  N  I  H  A  Q  W  P  E  S  Q  V  L  I  A  R  P  N  G  N  G  N  N  W  G  V

1281 ACGATCCAGCACAACGGCAACTGGACCTGGCCGACGGTCACCTGTACCGCGAACTGAGTTCCCGCCCCCAAAGGTGGCGC  1360
     T  I  Q  H  N  G  N  W  T  W  P  T  V  T  C  T  A  N  *

1361 GGCGGCTCCCGGCCG  1375
```

FIGURE 13

```
   1 TTCGGCAGCCTATTGACAAATTTCGTGAATGTTTCCCACACTTGCTCTGCAGACGGCCCCGCCGATCATGGGTGCACCGG   80

  81 TCGGCGGGACCGTGCTCCGACGCCATTCGGGGGTGTGCGCCTGCGGGCGCGGCGTCGATCCCGCGGGGACTCCCGCGGTT  160

 161 CCCTTTCCGTGTCCCTCTAATGGAGGCTCAGGCATGGGCGTGAACGCCTTCCCCAGACCCGGAGCTCGGCGGTTCACCGG  240
                                      M  G  V  N  A  F  P  R  P  G  A  R  R  F  T  G

 241 CGGGCTGTACCGGGCCCTGGCCGCGGCCACGGTGAGCGTGGTCGGCGTGGTCACGGCCCTGACGGTGACCCAGCCCGCCA  320
      G  L  Y  R  A  L  A  A  A  T  V  S  V  V  G  V  V  T  A  L  T  V  T  Q  P  A  S

 321 GCGCCGCGGCGAGCACGCTCGCCGAGGGTGCCGCGCAGCACAACCGGTACTTCGGCGTGGCCATCGCCGCGAACAGGCTC  400
       A  A  A  S  T  L  A  E  G  A  A  Q  H  N  R  Y  F  G  V  A  I  A  A  N  R  L
       <------------ #1696 peptide ------------><------- #1697 peptide ------><---

 401 ACCGACTCGGTCTACACCAACATCGCGAACCGCGAGTTCAACTCGGTGACGGCCGAGAACGAGATGAAGATCGACGCCAC  480
     T  D  S  V  Y  T  N  I  A  N  R  E  F  N  S  V  T  A  E  N  E  M  K  I  D  A  T
     ------ #1698 peptide ----------><------ # 1704 peptide ------------>

 481 CGAGCCGCAGCAGGGGCGGTTCGACTTCACCCAGGCCGACCGGATCTACAACTGGGCGCGCCAGAACGGCAAGCAGGTCC  560
      E  P  Q  Q  G  R  F  D  F  T  Q  A  D  R  I  Y  N  W  A  R  Q  N  G  K  Q  V  R

 561 GCGGCCACACCCTGGCCTGGCACTCGCAGCAGCCGCAGTGGATGCAGAACCTCAGCGGCCAGGCGCTGCGCCAGGCGATG  640
      G  H  T  L  A  W  H  S  Q  Q  P  Q  W  M  Q  N  L  S  G  Q  A  L  R  Q  A  M

 641 ATCAACCACATCCAGGGGGTCATGTCCTACTACCGGGGCAAGATCCCGATCTGGGACGTGGTGAACGAGGCGTTCGAGGA  720
     I  N  H  I  Q  G  V  M  S  Y  Y  R  G  K  I  P  I  W  D  V  V  N  E  A  F  E  D

 721 CGGAAACTCCGGCCGCCGGTGCGACTCCAACCTCCAGCGCACCGGTAACGATTGGATCGAGGTCGCGTTCCGCACCGCCC  800
      G  N  S  G  R  R  C  D  S  N  L  Q  R  T  G  N  D  W  I  E  V  A  F  R  T  A  R

 801 GCCAGGGGGACCCCTCGGCCAAGCTCTGCTACAACGACTACAACATCGAGAACTGGAACGCGGCCAAGACCCAGGCGGTC  880
      Q  G  D  P  S  A  K  L  C  Y  N  D  Y  N  I  E  N  W  N  A  A  K  T  Q  A  V
                                                                           <-----------

 881 TACAACATGGTGCGGGACTTCAAGTCCCGCGGCGTGCCCATCGACTGCGTGGGCTTCCAGTCGCACTTCAACAGCGGTAA  960
     Y  N  M  V  R  D  F  K  S  R  G  V  P  I  D  C  V  G  F  Q  S  H  F  N  S  G  N
     - #1703 peptide --->

 961 CCCGTACAACCCGAACTTCCGCACCACCCTGCAGCAGTTCGCGGCCCTCGGCGTGGACGTCGAGGTCACCGAGCTGGACA 1040
      P  Y  N  P  N  F  R  T  T  L  Q  Q  F  A  A  L  G  V  D  V  E  V  T  E  L  D  I

1041 TCGAGAACGCCCCGGCCCAGACCTACGCCAGCGTGATCCGGGACTGCCTGGCCGTGGACCGCTGCACCGGCATCACCGTC 1120
      E  N  A  P  A  Q  T  Y  A  S  V  I  R  D  C  L  A  V  D  R  C  T  G  I  T  V
         ^                                                              <--- # 1699 peptide

1121 TGGGGTGTCCGCGACAGCGACTCCTGGCGCTCGTACCAGAACCCGCTGCTGTTCGACAACAACGGCAACAAGAAGCAGGC 1200
     W  G  V  R  D  S  D  S  W  R  S  Y  Q  N  P  L  L  F  D  N  N  G  N  K  K  Q  A
     ------->
```

FIGURE 14

```
1201  CTACTACGCGGTGCTCGACGCCCTGAACGAGGGCTCCGACGACGGTGGCGGCCCGTCCAACCCGCCGGTCTCGCCGCCGC  1280
        Y  Y  A  V  L  D  A  L  N  E  G  S  D  D  G  G  G  P  S  N  P  P  V  S  P  P  P

1281  CGGGTGGCGGTTCCGGGCAGATCCGGGGCGTGGCCTCCAACCGGTGCATCGACGTGCCGAACGGCAACACCGCCGACGGC  1360
        G  G  G  S  G  Q  I  R  G  V  A  S  N  R  C  I  D  V  P  N  G  N  T  A  D  G

1361  ACCCAGGTCCAGCTGTACGACTGCCACAGCGGTTCCAACCAGCAGTGGACCTACACCTCGTCCGGTGAGTTCCGCATCTT  1440
        T  Q  V  Q  L  Y  D  C  H  S  G  S  N  Q  Q  W  T  Y  T  S  S  G  E  F  R  I  F

1441  CGGCAACAAGTGCCTGGACGCGGGCGGCTCCAGCAACGGTGCGGTGGTCCAGATCTACAGCTGCTGGGGCGGCGCCAACC  1520
        G  N  K  C  L  D  A  G  G  S  S  N  G  A  V  V  Q  I  Y  S  C  W  G  G  A  N  Q

1521  AGAAGTGGGAGCTCCGGGCCGACGGCACCATCGTGGGCGTGCAGTCCGGGCTGTGCCTCGACGCGGTGGGTGGCGGCACC  1600
        K  W  E  L  R  A  D  G  T  I  V  G  V  Q  S  G  L  C  L  D  A  V  G  G  G  T

1601  GGCAACGGCACGCGGCTGCAGCTCTACTCCTGCTGGGGCGGCAACAACCAGAAGTGGTCCTACAACGCCTGATCCCCGGC  1680
        G  N  G  T  R  L  Q  L  Y  S  C  W  G  G  N  N  Q  K  W  S  Y  N  A  *

1681  TGATCGACCCTAGTTGAGGCCGTCTCCGGTACGGCACCGTCGGACCGGAGGCGGTCCCTTGTTCGTCCAGGACGGAAGGA  1760

1761  CCGGTCTGAGCAGGCGCGGCGATCGGACACCATGGTGGGAGGCACGAAAGCGGGAGGGGGTCGTATTCCGAGACTCCGGG  1840

1841  AAGTGGAGGTGTTCCTCCACCTGA  1864
```

FIGURE 14 (CONTINUED)

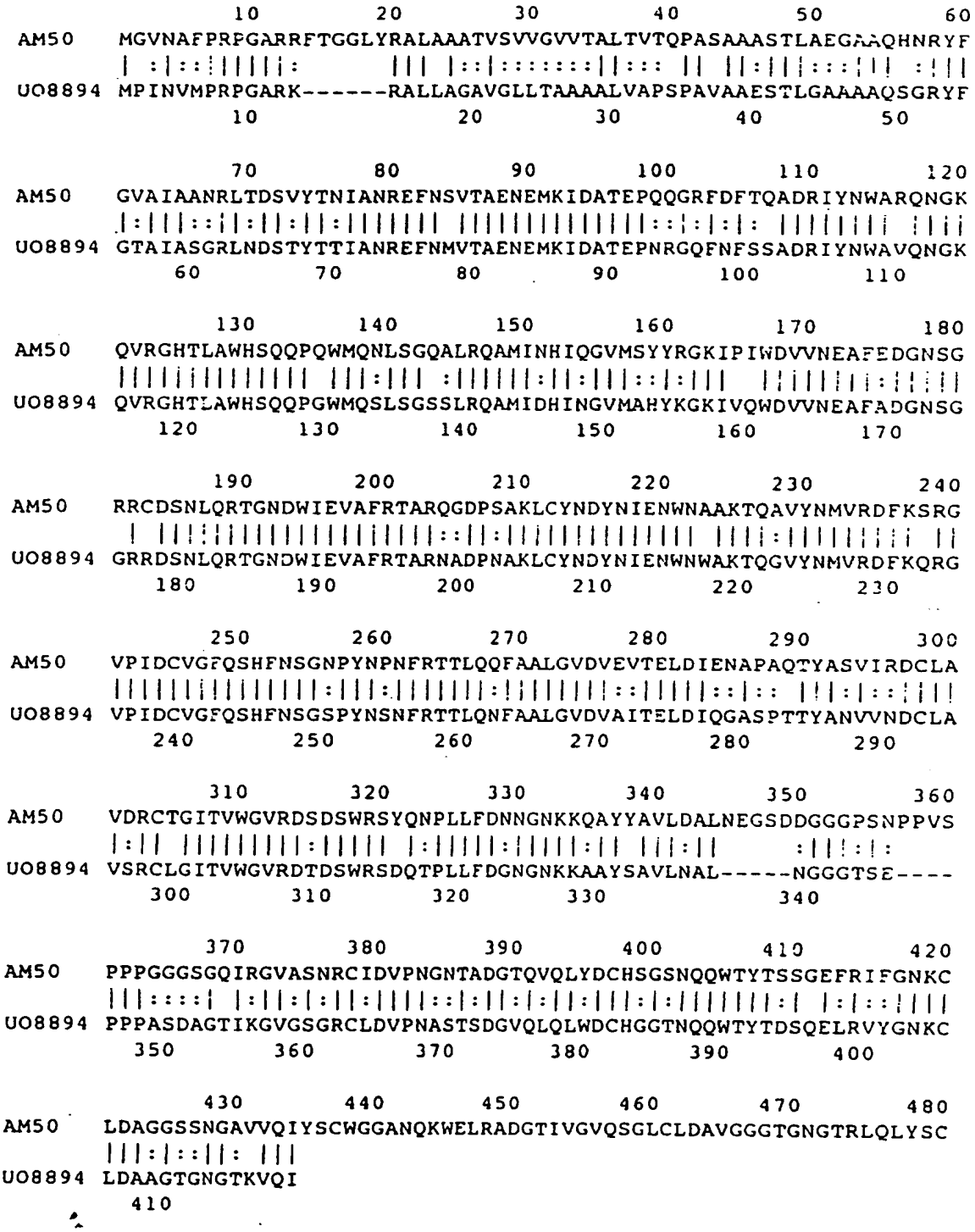
AM50 MGVNAFPRPGARRFTGGLYRALAAATVSVVGVVTALTVTQPASAAASTLAEGAAQHNRYF
UO8894 MPINVMPRPGARK------RALLAGAVGLLTAAAALVAPSPAVAAESTLGAAAAQSGRYF
AM50 GVAIAANRLTDSVYTNIANREFNSVTAENEMKIDATEPQQGRFDFTQADRIYNWARQNGK
UO8894 GTAIASGRLNDSTYTTIANREFNMVTAENEMKIDATEPNRGQFNFSSADRIYNWAVQNGK
AM50 QVRGHTLAWHSQQPQWMQNLSGQALRQAMINHIQGVMSYYRGKIPIWDVVNEAFEDGNSG
UO8894 QVRGHTLAWHSQQPGWMQSLSGSSLRQAMIDHINGVMAHYKGKIVQWDVVNEAFADGNSG
AM50 RRCDSNLQRTGNDWIEVAFRTARQGDPSAKLCYNDYNIENWNAAKTQAVYNMVRDFKSRG
UO8894 GRRDSNLQRTGNDWIEVAFRTARNADPNAKLCYNDYNIENWNWAKTQGVYNMVRDFKQRG
AM50 VPIDCVGFQSHFNSGNPYNPNFRTTLQQFAALGVDVEVTELDIENAPAQTYASVIRDCLA
UO8894 VPIDCVGFQSHFNSGSPYNSNFRTTLQNFAALGVDVAITELDIQGASPTTYANVVNDCLA
AM50 VDRCTGITVWGVRDSDSWRSYQNPLLFDNNGNKKQAYYAVLDALNEGSDDGGGPSNPPVS
UO8894 VSRCLGITVWGVRDTDSWRSDQTPLLFDGNGNKKAAYSAVLNAL-----NGGGTSE----
AM50 PPPGGGSGQIRGVASNRCIDVPNGNTADGTQVQLYDCHSGSNQQWTYTSSGEFRIFGNKC
UO8894 PPPASDAGTIKGVGSGRCLDVPNASTSDGVQLQLWDCHGGTNQQWTYTDSQELRVYGNKC
AM50 LDAGGSSNGAVVQIYSCWGGANQKWELRADGTIVGVQSGLCLDAVGGGTGNGTRLQLYSC
UO8894 LDAAGTGNGTKVQI

FIGURE 15A

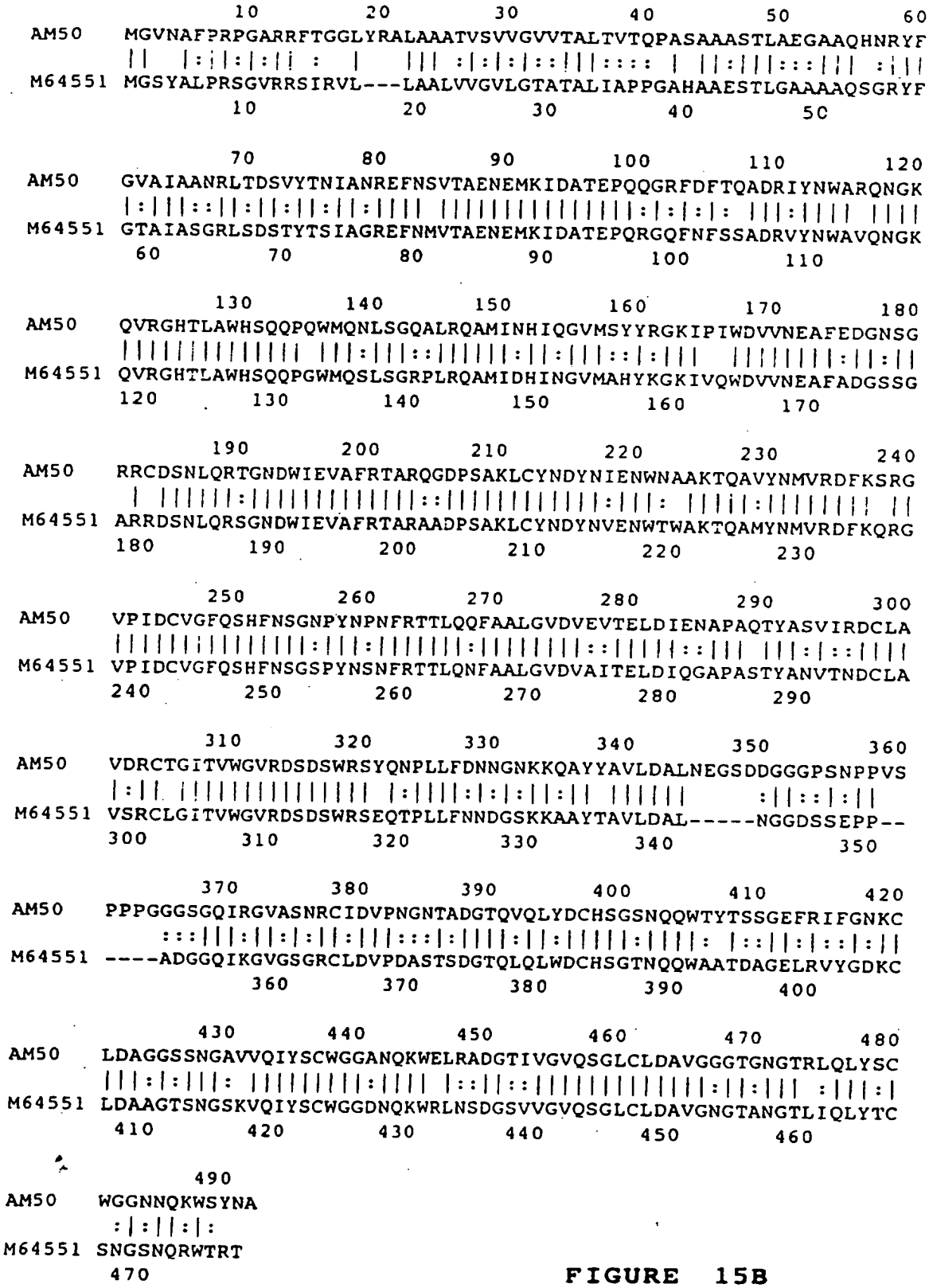
AM50 MGVNAFPRPGARRFTGGLYRALAAATVSVVGVVTALTVTQPASAAASTLAEGAAQHNRYF
M64551 MGSYALPRSGVRRSIRVL---LAALVVGVLGTATALIAPPGAHAAESTLGAAAAQSGRYF
AM50 GVAIAANRLTDSVYTNIANREFNSVTAENEMKIDATEPQQGRFDFTQADRIYNWARQNGK
M64551 GTAIASGRLSDSTYTSIAGREFNMVTAENEMKIDATEPQRGQFNFSSADRVYNWAVQNGK
AM50 QVRGHTLAWHSQQPQWMQNLSGQALRQAMINHIQGVMSYYRGKIPIWDVVNEAFEDGNSG
M64551 QVRGHTLAWHSQQPGWMQSLSGRPLRQAMIDHINGVMAHYKGKIVQWDVVNEAFADGSSG
AM50 RRCDSNLQRTGNDWIEVAFRTARQGDPSAKLCYNDYNIENWNAAKTQAVYNMVRDFKSRG
M64551 ARRDSNLQRSGNDWIEVAFRTARAADPSAKLCYNDYNVENWTWAKTQAMYNMVRDFKQRG
AM50 VPIDCVGFQSHFNSGNPYNPNFRTTLQQFAALGVDVEVTELDIENAPAQTYASVIRDCLA
M64551 VPIDCVGFQSHFNSGSPYNSNFRTTLQNFAALGVDVAITELDIQGAPASTYANVTNDCLA
AM50 VDRCTGITVWGVRDSDSWRSYQNPLLFDNNGNKKQAYYAVLDALNEGSDDGGGPSNPPVS
M64551 VSRCLGITVWGVRDSDSWRSEQTPLLFNNDGSKKAAYTAVLDAL-----NGGDSSEPP--
AM50 PPPGGGSGQIRGVASNRCIDVPNGNTADGTQVQLYDCHSGSNQQWTYTSSGEFRIFGNKC
M64551 ----ADGGQIKGVGSGRCLDVPDASTSDGTQLQLWDCHSGTNQQWAATDAGELRVYGDKC
AM50 LDAGGSSNGAVVQIYSCWGGANQKWELRADGTIVGVQSGLCLDAVGGGTGNGTRLQLYSC
M64551 LDAAGTSNGSKVQIYSCWGGDNQKWRLNSDGSVVGVQSGLCLDAVGNGTANGTLIQLYTC
AM50 WGGNNQKWSYNA
M64551 SNGSNQRWTRT

FIGURE 15B

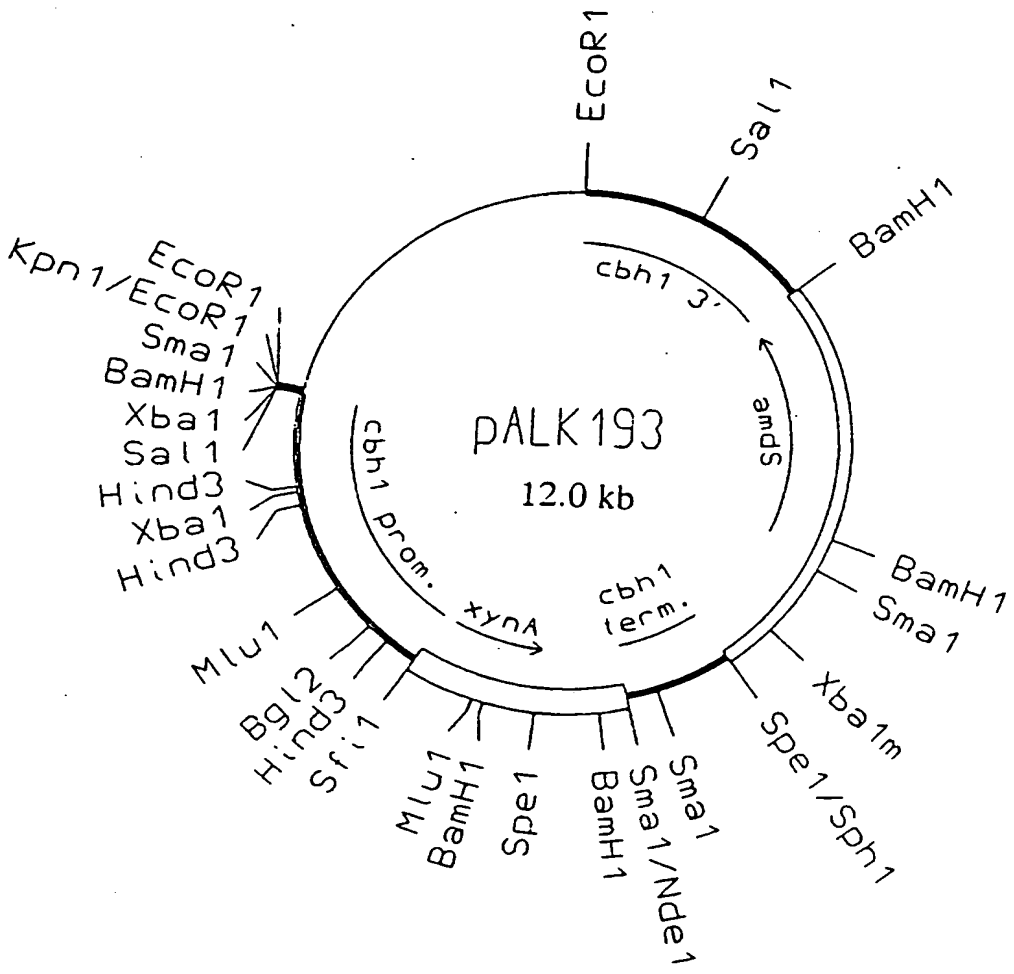
EcoR1
Sal 1
BamH1
cbh1 3'
pALK193
12.0 kb
spmds
BamH1
Sma 1
Xba1m
Spe1/Sph1
Sma1
BamH1
Sma1/Nde1
Spe1
Mlu1
BamH1
Bgl2
Hind3
Sfi 1
Mlu1
cbh1 prom.
xynA
cbh1 term.
EcoR1
Kpn 1/EcoR1
Sma 1
BamH1
Xba1
Sal 1
Hind3
Xba1
Hind3

FIGURE 16

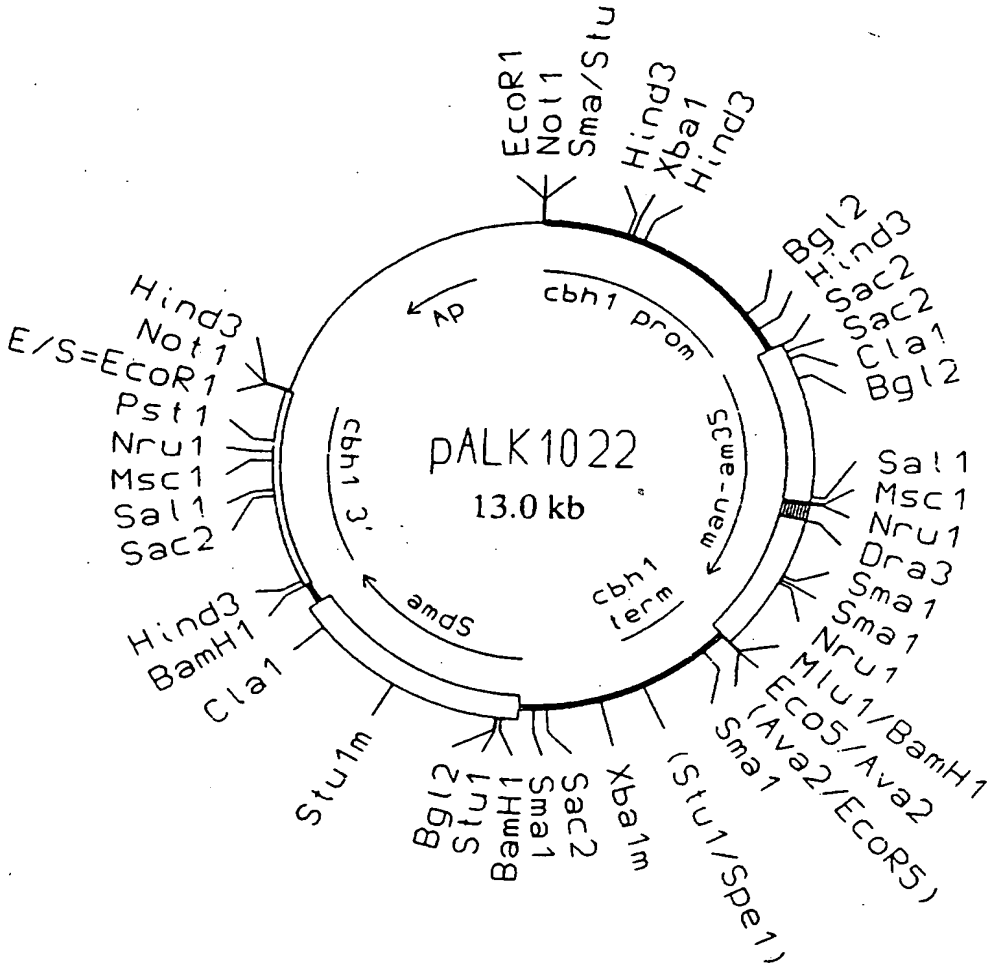
pALK1022
13.0 kb
cbh1 prom
man-amdS
cbh1 term
amdS
cbh1 3'
AP
EcoR1
Not1
Sma/Stu
Hind3
Xba1
Hind3
Bgl2
Hind3
Xba1
Sac2
Sac2
Cla1
Bgl2
Sal1
Msc1
Nru1
Dra3
Sma1
Sma1
Nru1
Mlu1/BamH1
ECO5/Ava2
(Ava2/ECOR5)
Sma1
(Stu1/Spe1)
Xba1m
Sac2
Sma1
BamH1
Stu1
Bgl2
Stu1m
Cla1
BamH1
Hind3
Sac2
Sal1
Msc1
Nru1
Pst1
E/S=ECoR1
Not1
Hind3

FIGURE 17

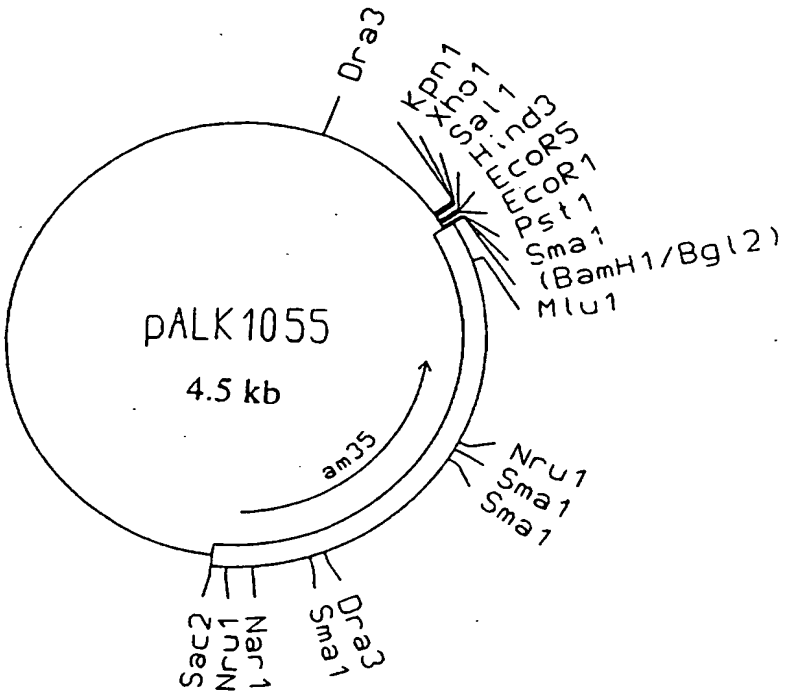
pALK1055
4.5 kb
am35
Dra3
Kpn1
Xho1
Sal1
Hind3
EcoRS
EcoR1
Pst1
Sma1
(BamH1/Bgl2)
Mlu1
Nru1
Sma1
Sma1
Sac2
Nru1
Nar1
Sma1
Dra3

FIGURE 18

DNA SEQUENCES OF THE FUSIONS BETWEEN *MAN1* CORE/HINGE AND AM35 GENE

pALK945

MAN...T GGT CGC *GAC ACC ACC...AM35* (SEQ ID NO: 11:)
G R *D T T* (SEQ ID NO: 12:)
man1 sequence AM35 sequence

pALK948

MAN...T GGT CGC GAC AAG CGC *GAC ACC ACC...AM35* (SEQ ID NO: 13:)
G R D K R *D T T* (SEQ ID NO: 14:)
KEX2-linker
man1 sequence AM35 sequence

pALK1021

MAN...T GGC CAG TGT GGA GGT *GAC ACC ACC ATC ACC CAG AAC...AM35*
G Q C G G *D T T I T Q N*
man1 sequence AM35 sequence
(SEQ ID NO: 15:)
(SEQ ID NO: 16:)

pALK1022

MAN...T GGC CAG TGT GGA GGT CGC GAC AAG CGC *GAC ACC ACC...AM35*
G Q C G G R D K R *D T T*
KEX-linker
man1 sequence AM35 sequence

(SEQ ID NO: 17:)
(SEQ ID NO: 18:)

FIGURE 19

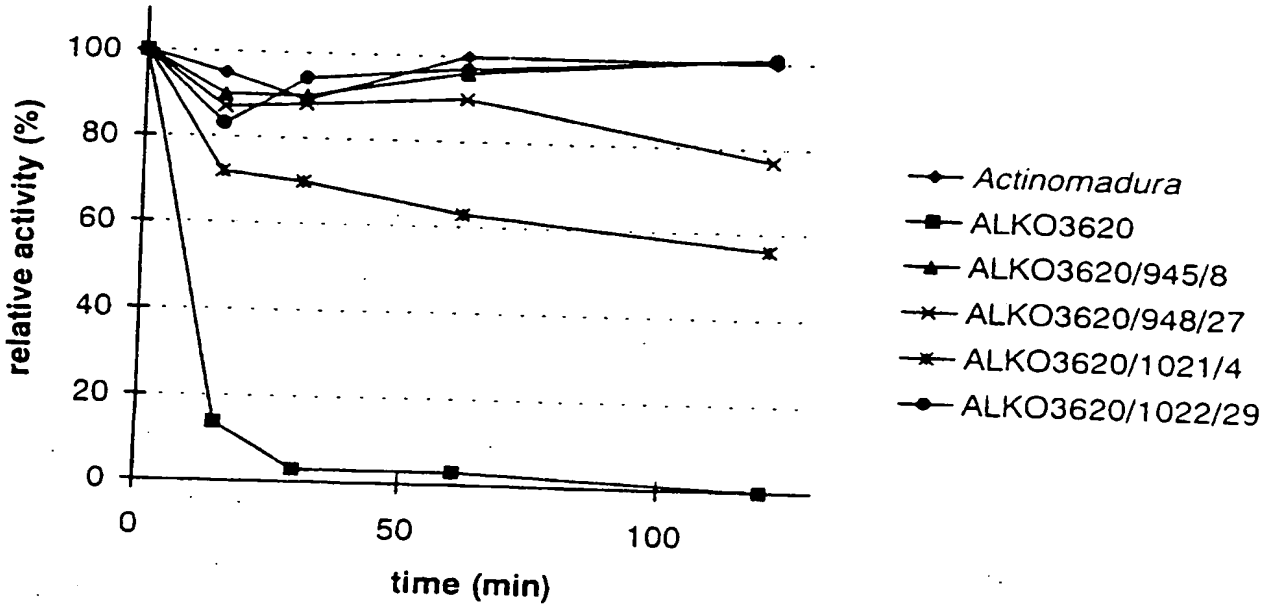
relative activity (%)
100
80
60
40
20
0
0
50
100
time (min)
Actinomadura
ALKO3620
ALKO3620/945/8
ALKO3620/948/27
ALKO3620/1021/4
ALKO3620/1022/29

FIGURE 20

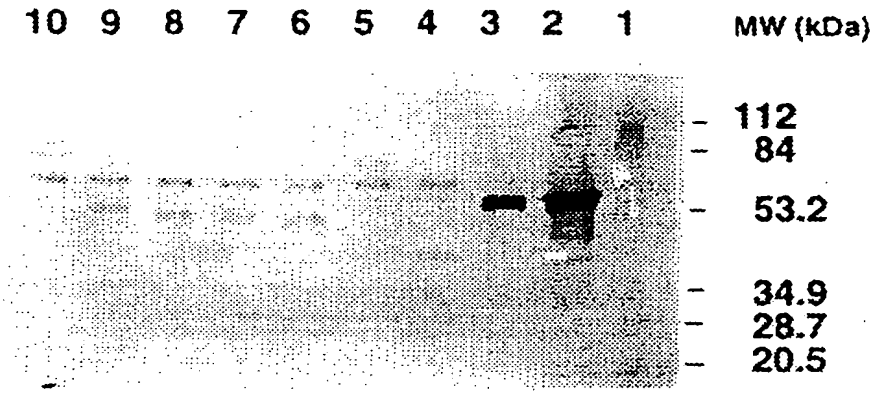

FIGURE 21

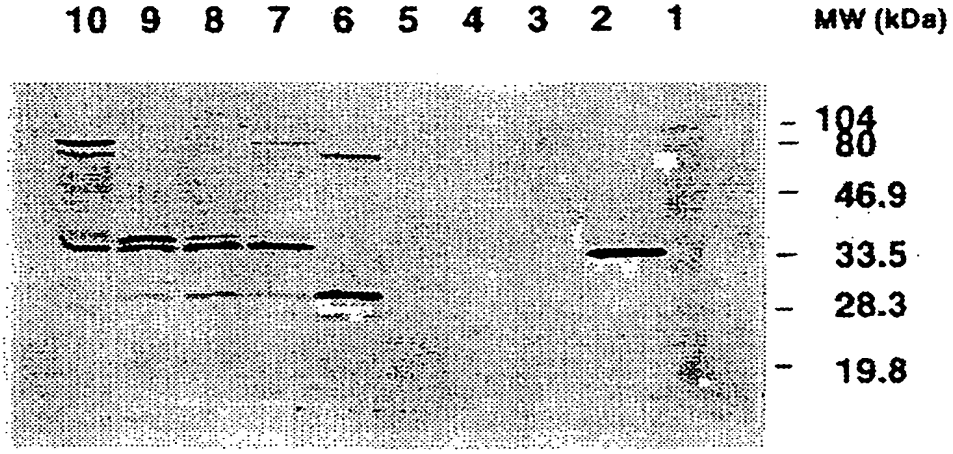

FIGURE 22

LANE

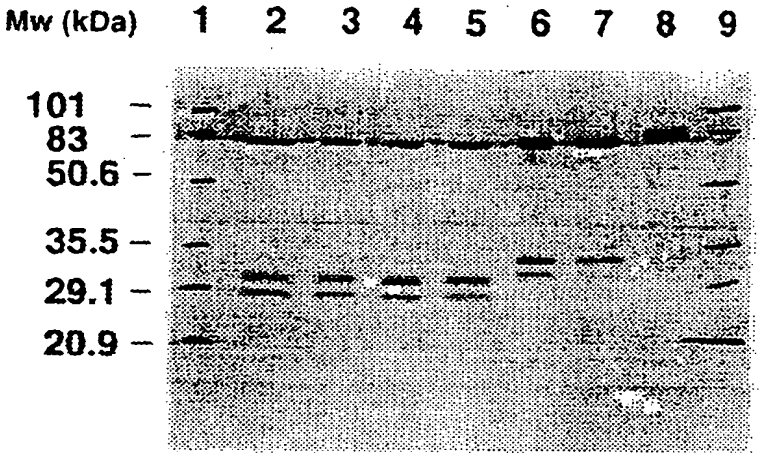

FIGURE 23A

LANE

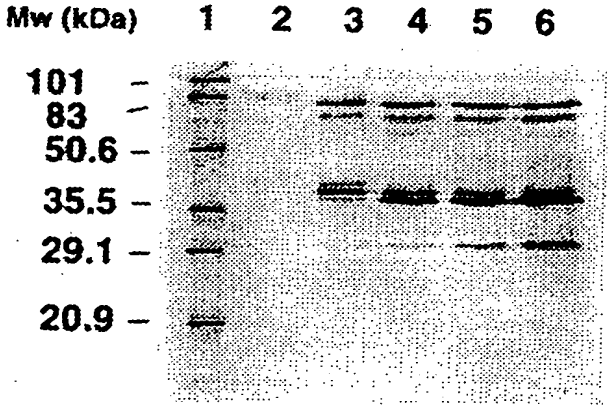

FIGURE 23B

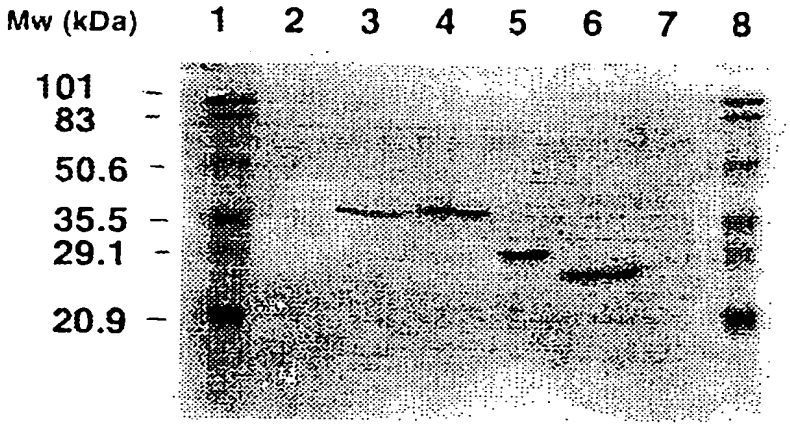

FIGURE 24

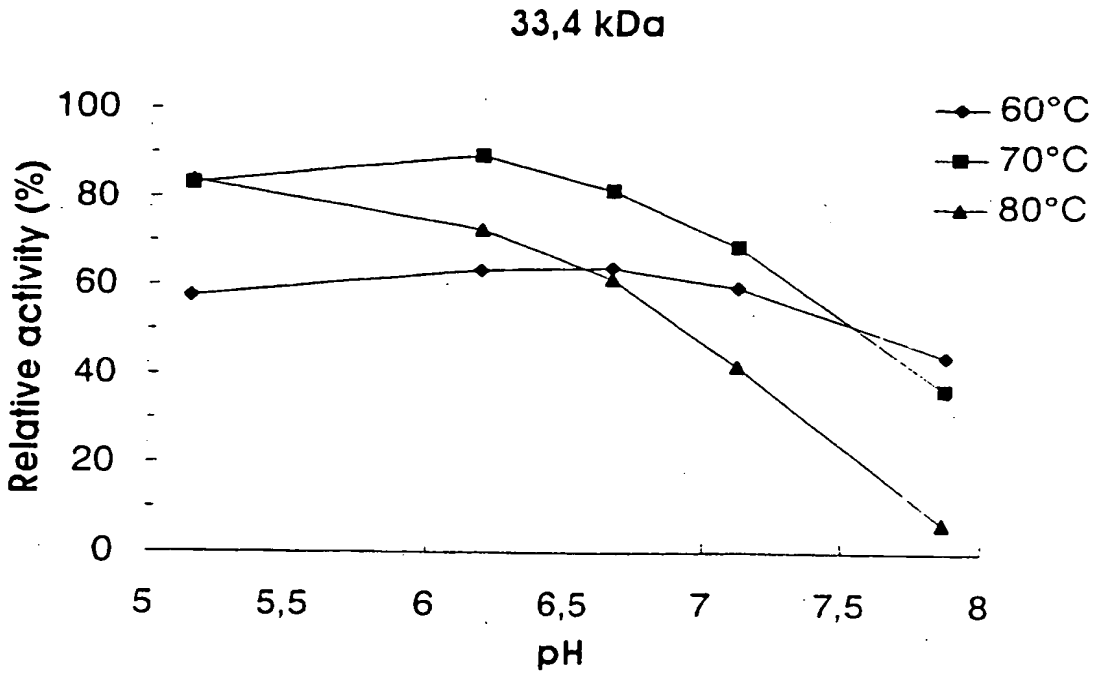

FIGURE 25A

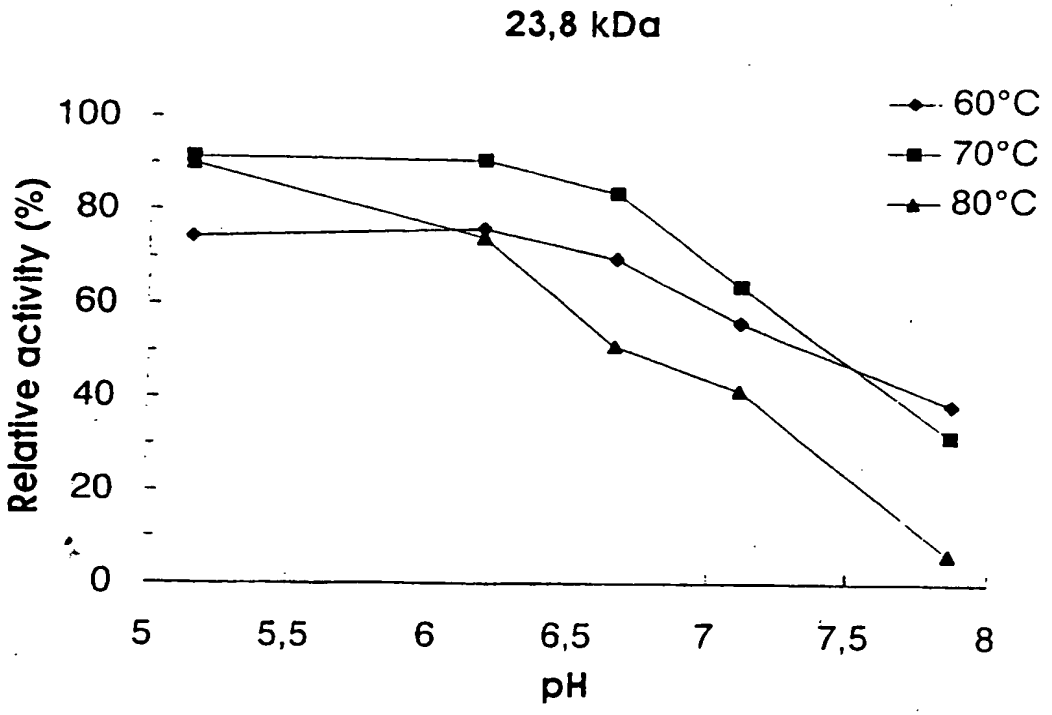

FIGURE 25B

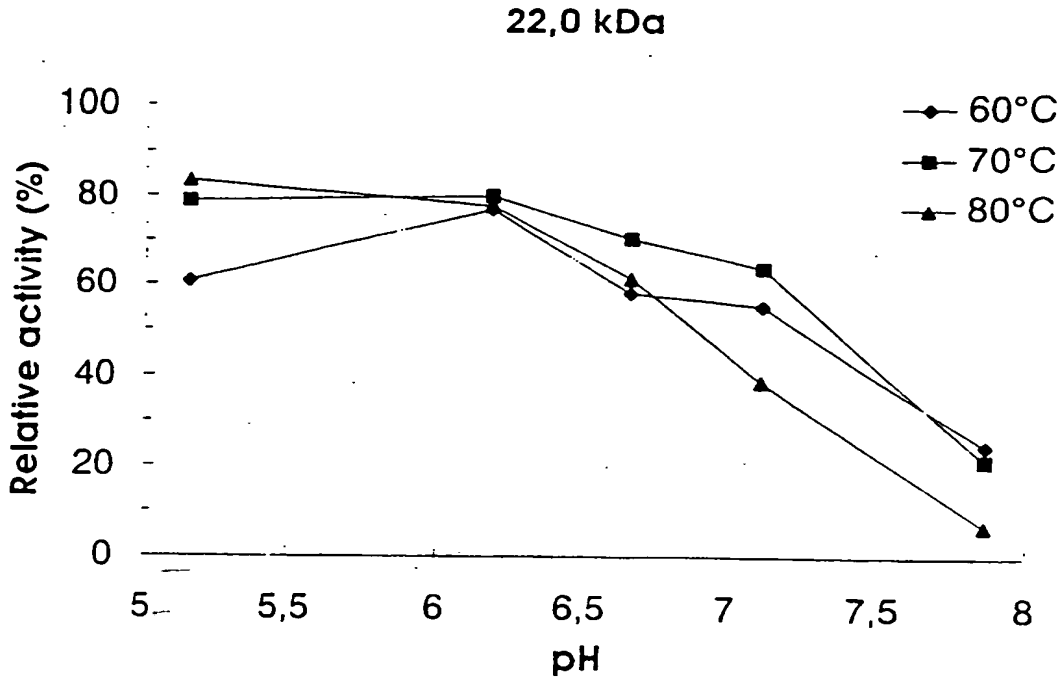

FIGURE 25C

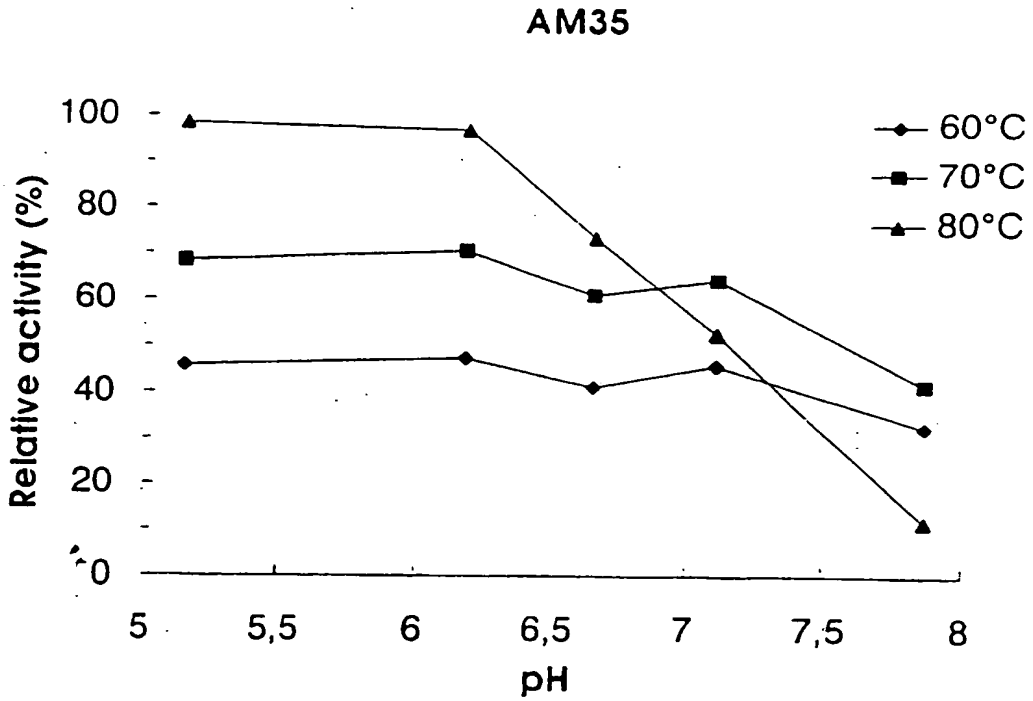

FIGURE 25D

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 97/00037

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/80, C12N 9/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, BIOSIS, MEDLINE, DBA, CA, EMBL/GENBANK/SWISSPROT/DDBJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 154, MEDLINE, Dialog accession no. 07407541, Medline accession no. 91207693, Punt PJ et al: "Intracellular and extracellular production of proteins in Aspergillus under the control of expression signals of the highly expressed Aspergillus nidulans gpdA gene"; & J Biotechnol (NETHERLANDS) Jan 1991, 17 (1) p19-33	1-28
X	EP 0215594 A2 (GENENCOR INC.), 25 March 1987 (25.03.87), page 6, line 9	1-28
X	WO 8901969 A1 (NOVO INDUSTRI A/S), 9 March 1989 (09.03.89), page 10, line 16 - line 29; page 12, line 12 - line 13	1-28

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 May 1997	08 -05- 1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI 97/00037

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0238023 A2 (NOVO INDUSTRI A/S), 23 Sept 1987 (23.09.87), page 6, line 6 - line 8 --	1-28
A	WO 9512668 A1 (CORNELL RESEARCH FOUNDATION, INC.), 11 May 1995 (11.05.95), the whole document, see expecially sequences	1-16,25
X	-- --------	17-24,26-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/FI 97/00037

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		AU 607398 B	07/03/91
		AU 6200886 A	05/03/87
		DE 3650202 D,T	01/06/95
		EP 0625577 A	23/11/94
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		JP 62175183 A	31/07/87
		US 5364770 A	15/11/94
		US 5578463 A	26/11/96
WO 8901969 A1	09/03/89	DE 3886221 D,T	19/05/94
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		SE 0383779 T3	
		JP 4503150 T	11/06/92
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		DE 3788524 D,T	11/05/94
		DK 169134 B	22/08/94
		EP 0489718 A	10/06/92
		ES 2061446 T	16/12/94
		IE 63169 B	22/03/95
		JP 7051067 A	28/02/95
		JP 62272988 A	27/11/87
WO 9512668 A1	11/05/95	EP 0728197 A	28/08/96
		FI 961885 A	03/07/96